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Tailored peptides: the synthesis and conformational behaviour of partially modified retro-inverso peptides

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TAILORED PEPTIDES: THE SYNTHESIS AND CONFORMATIONAL BEHAVIOUR OF PARTIALLY MODIFIED RETRO-INVERSO PEPTIDES.

Submitted by

Matthew David Fletcher

for the degree of PhD

of the University of Bath

1996

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Abstract

Synthetic methodology is described that permits access to partially modified retro-inverso (PMRI) peptides containing the structural motif $[Xaa\psi(NHCO)Gly]_n$, $n \geq 2$. Such PMRI peptides were expected to be predisposed to β-sheet formation on the basis of molecular modelling studies.

The PMRI dipeptides Boc-Xaaψ(NHCO)Gly-OEt (Xaa = Gly, Val, and Phe) were synthesised using a Goldschmidt and Wick type procedure. *C*- and *N*-terminal deprotections of these PMRI dipeptides were accomplished by saponification and acidolysis, respectively.

PMRI dipeptides were coupled to yield Boc-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-OEt. EDC / HOSu coupling was the method of choice to yield Boc-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-OEt (Xaa = Val and Phe). For the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, isolation of the active ester and subsequent coupling was preferred. Carbonate mixed anhydride couplings were only successful between *gem*-diamino residues and non-malonyl residues. Use of these coupling (and deprotection) procedures yielded Boc-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-OEt (Xaa = Gly and Val); but failed to furnish Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt. Poor solubility of all PMRI peptides containing four or more residues dogged the syntheses and could only be tackled by the use of highly polar solvents.

PMRI dipeptides Fmoc-Xaaψ(NHCO)Gly (Xaa = Gly and Val) were obtained from the corresponding *N*-Boc PMRI dipeptides. SPS of PMRI peptides was demonstrated using Fmoc-Glyψ(NHCO)Gly, but insolubility prohibited purification of the product, Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2.

Solubility problems necessitated the use of $DMSO_{d6}$ as solvent for NMR studies. Under these conditions no secondary structure was detected. CD spectra of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt suggested some defined secondary structure of the -*m*Gly^2-Pro^3-Gly^4- sequence.

Brand new you're retro[1]

A Man would do nothing, if he waited until he could do it so well that no one would find fault with what he has done.

Cardinal Newman.

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"Praise God from whom all blessings flow."

Dedicated to those whose love has nurtured and sustained me: Rachael, Freda, Michael, Madeleine, Jonathan, Helen, Jeff and Charlotte.

Abbreviations

A	Denotes a figure in the **Appendix**.
ACE	Angiotensin converting enzyme.
Adoc	Adamantyloxycarbonyl.
Aib	α-Amino-*iso*-butyric acid.
AM1	Austin model 1.
AN	Electron-acceptor number.
Bn	Benzyl group or benzene.
Boc	*tert*-Butoxycarbonyl.
Bom	Benzyloxymethyl.
BOP	1-Benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate.
b.p.	Boiling point.
br	Broad (preventing measurement of *J* when pertaining to ^{1}H NMR).
BSA	*O*,*N*-Bis(trimethylsilyl)acetamide.
Bt	Benzotriazole.
cat	Catalytic quantity.
CCK	Cholecystokinin.
CD	Circular dichroism.
C.I.	Chemical ionisation.
CNS	Central nervous system.
conc	Concentrated.
COSY	2D correlated spectroscopy.
Da	Dalton.
DABCO	1,4-Diazabicyclo[2.2.2]octane.
DCC	Dicyclohexylcarbodiimide.
DCM	Dichloromethane.
DCU	*N*,*N*'-Dicyclohexylurea.
DEPT	Distortionless enhancement by polarisation transfer.

DIBAL	Di-*iso*-butylaluminium hydride.
DIPCDI	Di-*iso*-propylcarbodiimide.
DIPEA	Di-*iso*-propylethylamine.
DIPU	Di-*iso*-propyl urea.
DMF	*N*,*N*-Dimethylformamide.
DMAP	4-Dimethylaminopyridine.
DMSO	Dimethyl sulfoxide.
DN	Electron-donor number.
DPPA	Diphenylphosphoryl azide.
DSC	*N*,*N*'-Disuccinimidyl carbonate.
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide.
E.I.	Electron impact.
FAB	Fast atom bombardment.
Fmoc	9-Fluorenylmethyloxycarbonyl.
For	Formyl.
Glp	Pyroglutamic acid.
HBTU	*O*-Benzotriazolyl-tetramethylisouronium hexafluorophosphate.
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol.
HIV	Human immunodeficiency virus.
HMPA	Hexamethyl phosphoramide.
HOBt	*N*-Hydroxybenzotriazole.
HOSu	*N*-Hydroxysuccinimide.
HPLC	High performance liquid chromatography.
IBTFA	Iodobenzene bis(trifluoroacetate).
IC_{50}	Median inhibitory concentration.
imid	Imidazole.
K_i	Inhibition constant.
LDA	Lithium di-*iso*-propylamide.
lit.	Literature.
min	Minor conformer.

MMA	*N*-Methylmercaptoacetamide.
MNP	(2-Methyl-2-*o*-nitrophenoxy)-propionyl.
m.p.	Melting point.
m.s.	Mass spectrometry.
Mtr	4-Methoxy-2,3,6-trimethylbenzenesulfonyl.
NBA	*m*-Nitrobenzyl alcohol.
NBS	*N*-Bromosuccinimide.
NK-1	Neurokinin / substance P receptor 1.
NMM	*N*-Methylmorpholine.
NMP	*N*-methyl-2-pyrrolidinone.
NMR	Nuclear magnetic resonance.
NOE	Nuclear Overhauser effect.
NOESY	2D nuclear Overhauser and exchange spectroscopy.
NT	Neurotensin.
nr	Not reported.
O/N	Overnight.
OPcp	Pentachlorophenyl.
OPfp	Pentafluorophenyl.
PAL	Peptide amide linker = 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy]-valeric acid.
PEG	Polyethylene glycol.
PG	Protecting group.
Pip	Piperidine.
PMRI	Partially modified retro-inverso.
PS	Polystyrene.
py	Pyridine.
Reflux	Heated under reflux.
RPHPLC	Reverse phase high performance liquid chromatography.
SAR	Structure activity relationship.
SCF-MO	Self consistent field molecular orbital.

SDS	Sodium dodecyl sulfate.
SPS	Solid phase synthesis.
TBAF	Tetrabutylammonium fluoride.
TBDMS	*tert*-Butyldimethylsilyl.
TBDMSOTf	*tert*-Butyldimethylsilyl trifluoromethanesulfonate.
TEBA	Triethylbenzylammonium chloride.
tech.	Technical grade.
TES	Triethylsilane.
TFA	Trifluoroacetic acid or trifluoroacetyl.
TFAA	Trifluoroacetic anhydride.
TFE	2,2,2-Trifluoroethanol.
THF	Tetrahydrofuran.
TLC	Thin layer chromatography.
TMEDA	*N*,*N*,*N*',*N*'-Tetramethylethylenediamine.
TMS	Trimethylsilyl or tetramethylsilane (in the context of NMR).
tol	Toluene.
Ts	Tosyl (*p*-toluenesulfonyl).
VT	Variable temperature.
Xaa	Unspecified α-amino acid.
Yaa	Unspecified α-amino acid.
Z	Benzyloxycarbonyl.
Zaa	Unspecified α-amino acid.

Notes On Nomenclature

IUPAC nomenclature and symbolism for peptides and peptide analogues is used.[2] Thus a dash, "-", represents a covalent bond (*c.f.* figure 4). Conversely a dot, ".", is used in this thesis to indicate salt formation. Therefore *N*-trifluoroacetylglycine, for example, would be represented as TFA-Gly, whereas TFA.Gly would represent glycinium trifluoroacetate.

The ψ (Greek psi) notation for amide bond surrogates indicates that the amide bond between the two residues is replaced by the unit in brackets following the ψ, *e.g.* -GlyΨ(NHCO)Gly- represents a glycylglycyl segment wherein the peptide bond is reversed: figure 1.

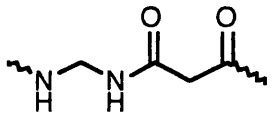

Figure 1: -GlyΨ(NHCO)Gly-.

For partially modified retro-inverso (PMRI) pseudopeptides the *g* / *m* / *r* prefix system is also used in the literature: *g*Xaa symbolises the *gem*-diamino alkyl analogue of the indicated amino acid residue, *m*Xaa symbolises the malonyl residue corresponding to the indicated amino acid residue, and *r*Xaa symbolises the reversed amino acid residue, *i.e.* -COCHRNH- rather than the conventional -NHCHRCO- direction. Thus, -*g*Gly-*m*Gly- corresponds to the structure shown in figure 1. This system of nomenclature is only used in this thesis to refer to PMRI peptides where the ψ notation is inappropriate, *e.g.* to refer to isolated residues.

Chemical Abstracts names PMRI pseudopeptides as propanoic acid derivatives. Thus, the PMRI pseudodipeptide PheΨ(NHCO)Gly is named (*R*)-3-[(1-amino-2-phenylethyl)-amino]-3-oxo-propanoic acid. This system of nomenclature rapidly becomes cumbersome and is not used in this thesis.

The following terms are used in this thesis with the given meaning.

- "Backbone": a homomeric*, homodetic† peptide "backbone" has three repeating units, the amide nitrogen, α-carbon, and amide carbonyl, see figure 2. R is referred to as the side chain. The backbone dihedral angles (ϕ_i,ψ_i), describe the conformation of the backbone at residue i.[3]‡

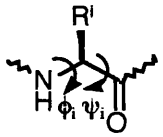

Figure 2: Peptide backbone residue i.

- "Cycloretro-enantiomer": an isomer of a cyclic compound in which the sequence is reversed and each residue is inverted.

- "End group modified retro-inverso isomer / peptide": a retro-inverso isomer in which the peptide chain end groups are altered to better correspond with those of the parent peptide.

- "Modified peptide": a peptide with some of its peptide functional groups replaced by other groups, but which retains properties analogous to the original peptide.

- "Partially modified retro-inverso (PMRI) peptide": an isomer of a linear peptide in which some of the peptide bonds are reversed and the chirality of the amino acids in the reversed section is inverted.

---

* Consisting of similar monomers, *i.e.* α-amino acids.

† Consisting of α-amino acid residues solely in eupeptide linkages (*i.e.* amide bonds formed between C^1 of Xaa^i and N^2 of Yaa^{i+1}).

‡ In this thesis the corresponding dihedral angles of both *gem*-diamino residues and malonyl residues are labelled (ϕ,ψ), but in other literature the labels (ϕ,ϕ') and (ψ,ψ') or (ϕ_1,ϕ_2) and (ψ_1,ψ_2) respectively, are used.

- "Peptidomimetic": a molecule with some or all peptide functional groups replaced by other groups, yet which exhibits properties analogous to a (or many) peptide(s).

- "Pseudopeptide": a peptide analogue with a backbone modification.

- "Retro-inverso peptide": an isomer of a linear peptide in which the direction of the amino acid sequence is reversed and the chirality of each amino acid is inverted.

- "Retro-isomer": an isomer (of a peptide) in which the direction of the amino acid sequence is reversed.

- "Surrogate": an unnatural replacement for a natural entity, *e.g.* "CH=CH" is an amide bond surrogate.

The following numbering system is used for the residues of PMRI pseudopeptides. Residues are numbered from the *N*-terminus, see figure 3.

Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt
1 2 3 4 5 6

Figure 3: Residue numbering.

This nomenclature is extended, as follows, to refer unambiguously to the component atoms of the residues. NHs are numbered as Val^{1}NH (or BocNH, see figure 4), Val^{1}ψNH, *etc*; side chains and α-carbons are numbered by residue; carbonyls are numbered as Gly^{2}ψCO, Gly^{2}CO, *etc*: see figure 4.

The terms *cis* and *trans* are used in this thesis to describe the configuration of amide bonds in accord with their general use in peptide and protein chemistry, *i.e.* the amide bond depicted in figure 1 is described as being in the *trans* configuration. Formally, the amide bond depicted in figure 1 would be described as the *s-cis* (or *Z*) form.

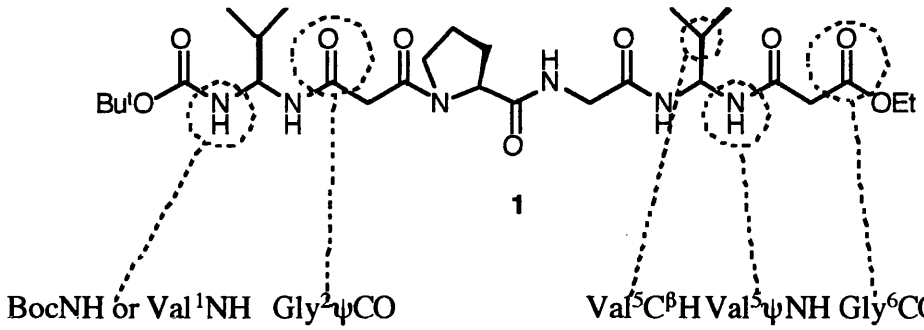

Figure 4: Atom and functional group labelling illustrated for Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1**.

Chapter One: Introduction

Peptides occur throughout nature in a wide range of roles. They act as extracellular messengers, *i.e.* hormones, neurotransmitters and neuromodulators, in plants and animals and thus influence such vital functions as metabolism, immune defence, respiration and reproduction. They carry out intracellular functions, *e.g.* the antioxidant and transport tripeptide glutathione. Indeed, they are essential to virtually every biochemical process. Peptides are also implicated in the appearance or maintenance of various diseases, *e.g.* the plaques associated with Alzheimer's disease.[4] Peptides find other structural applications, *e.g.* as the cross-links in the peptidoglycan cell walls of bacteria; but the structural domain is more properly the realm of polypeptides and proteins. More esoteric roles for peptides include mushroom toxins, components of snake venoms, and antifreeze in fish.

This broad spectrum of activity has attracted much attention to peptides from bioorganic, medicinal and polymer chemists, to name but a few.

1.1 *Why Modify?-*

The desire to use peptides as pharmaceuticals is the major incentive for modification, or tailoring. The pharmacological properties of most peptides preclude their use as drugs. The mammalian body presents many barriers to the entry of macromolecules and thus peptides fall foul of poor absorption, because they do not readily pass across biological membranes; swift metabolism by proteolytic enzymes; and rapid excretion through the liver and kidneys.[5-7] These barriers result in peptides suffering from low bioavailability and short biological half-lives. In addition there is the problem of specificity: peptide receptors can be widely distributed in an organism and their stimulation results in a variety of desired and undesired effects, especially when the peptide is conformationally flexible and hence able to interact with alternative receptors.[6]

The aim of peptide modification is to determine the structure activity relationships of endogenous peptides and to produce analogues which can overcome the barriers and problems described above, while retaining selected activity (*i.e.* specific receptor

agonists). Conversely, receptor antagonists and enzyme inhibitors are also desirable targets attainable through peptide modification.[6,8,9]

The realisation of these goals is aided by the simultaneous programme of discovery and development of peptidomimetics from leads other than the endogenous peptides whose action is of interest. Various definitions of peptidomimetics or peptide mimetics (the terms are used interchangeably) exist in the literature:

- Wiley and Rich — "Chemical structures designed to convert the information contained in peptides into small nonpeptide structures."[10]

- Giannis and Kolter — "A compound that, as a ligand of a receptor, can imitate or block the biological effect of a peptide at the receptor level."[6]

- Moore — "A molecule that mimics the biological activity of a peptide but is no longer peptidic in chemical nature. The term is... sometimes used to describe molecules that are no longer completely peptidic in nature, such as pseudopeptides... but a strict definition is a molecule that no longer contains any peptide bonds... and has a molecular weight of less than 700 Da."[11]

- Morgan and Gainor — "Structures which serve as appropriate substitutes for peptides in interactions with receptors and enzymes."[12]

- Gante — "[A] chemical 'Trojan horse'... A substance having a secondary structure as well as other structural features analogous to that of the original peptide, which allows it to displace the original peptide from receptors or enzymes. As a result the effects of the original peptide are inhibited (antagonist, inhibitor) or duplicated (agonist)."[8]

- Kemp — "Elements which mimic the structure of natural peptide components."[13]

Olson *et al.* do not offer a formal definition, but their comments are informative: "The term... 'peptidomimetics' [has been] utilized... to describe compounds discovered through a variety of research strategies. Indeed, even compounds identified by random screening and subsequently optimized through structural modification have been termed peptidomimetics if the initial lead was found in an assay in which the natural ligand is a peptide or protein. The field of enzyme inhibitors uses peptide mimetics terminology for the replacements of segments of peptide-based substrates and inhibitors... The broad use of the term "peptide mimetics" is unavoidable, but the advocates of rational design do not favor its use to describe compounds found by screening."[14]

Veber recently coined the term "peptide limetic" (a contraction of ligand mimetic) to distinguish compounds (discovered by screening) so divergent from peptides "that specific connections are no longer recognizable" but which exhibit activity in endogenous peptide assays, from peptidomimetics more rationally derived.[15] This term finds sporadic use in the literature, with its own twists of definition.[10]

These definitions and comments bring to light the importance of the screening of natural products (and other compound collections) in the discovery and development of peptidomimetics.[6,10,12,16] The natural products (and other compounds) unearthed by screening may be peptidic or non-peptidic, and are particularly valuable in the search for antagonistic peptidomimetics and enzyme inhibitors.[6,10,12,16]

The rather broad definition of a peptidomimetic conformed to here is: a molecule with some or all peptide functional groups replaced by other groups, yet which exhibits properties analogous to a (or many) peptide(s).

Thus, with this definition, a modified peptide may also be described as a peptidomimetic, but a peptidomimetic is not necessarily a modified peptide.

There are sources of peptidomimetics other than screening and modification starting from an endogenous peptide. The recent ascendance of peptide libraries is founded predominantly on their promise of leads for peptidomimetics.[17-20] Indeed, attention is now also directed upon the development of peptidomimetic libraries, which offer further molecular diversity.[21-26]

A different approach is that of Greene and co-workers, in which peptidomimetics are developed from the complimentarity determining region of monoclonal antibodies raised against receptors of interest.[27]

This antibody approach and Kemp's distinct definition of peptidomimetics,[13] reveal motives for modification which are less firmly in the medicinal chemistry camp. These motives are the study of molecular recognition[27] and investigations into protein folding.[28] The aspiration to an understanding of protein folding inspires much work on small molecules (*e.g.* the studies of Gellman and co-workers[29]) and modified peptides,[28] which does not sit easily within the more medicinal chemical definitions of peptidomimetics, but which ought to be considered in this context [see section **1.5.3***(b)(iii)(2)* and *(iv)(2)*].

1.2 *The Range of Peptide Modifications and Peptidomimetics.-*

As might be expected from the variety of definitions, the range of peptidomimetics is wide, and different reviewers adopt different modes of classification. Thus peptidomimetics may be classified according to origin,[10,12] activity,[8,10,11,30] secondary structure,[31-35] topographical considerations,[36] that part of the endogenous peptide which is modified,[6,8] or which peptide torsion angles are constrained.[32] Modification can be made at any point in a peptide and modifications can be applied in any combination:[37,38] amino acids can be deleted, or added / replaced (by coded or noncoded alternatives);[39] short-[40] or long-range cyclisations[41] may be applied, through the side chains or backbone; backbone peptide bonds can be replaced with surrogates;[38,42] or the backbone may be replaced altogether by a novel scaffold,[8,16] or alternative backbone [see section **1.5.3***(b)(iii)(2)*]. An overview of modifications, and combinations thereof, used in the development of therapeutic peptides, is provided by Dutta, in his review of the design of peptide-based drugs.[43] Rational, general strategies for the use of modifications are under development: both Hruby[44,45] and Marshall[46] have recently published their approaches for the use of modifications (with the emphasis on conformationally constrained and chimeric, novel amino acids) to produce selective, biologically active, peptide and peptidomimetic ligands.

The subject of this thesis is a particular peptide bond modification, and its influence on the conformation of peptides into which it is incorporated. Therefore an attempt to tidily classify the world of peptidomimetics is beyond the scope of this introduction (the interested reader is referred to the reviews already cited and the annual Chemical Society specialist reports[47]), and further discussion is restricted to peptide bond surrogates in general, and the retro-inverso modification in particular.

1.3 *Peptide Bond Surrogates.-*

Peptide bond surrogates are amide look-alikes used to replace backbone peptide bonds. Generally these surrogates are isosteric and / or isoelectronic with the peptide bond, and indeed, are often referred to as isosteres. However, the isosteric and / or isoelectronic character of the surrogates is not always obvious, nor even necessary for biological activity.

Peptide bond surrogates are used to investigate the role and function of backbone peptide bonds, and to modify the properties of the parent peptides. In his peptide backbone modifications review of 1983,[38] Spatola raised these questions of interest (with particular reference to peptide hormone analogues):

- Is there a functional role for the peptide bond itself? Or does the backbone merely serve to orient and align the essential side chain residues?

- To what extent are the alignment, bond lengths, and stereochemistry of the peptide backbone critical for the resulting biological function?

- How does the modification of the peptide backbone affect the resistance (as measured by biological half-lives) toward enzymatic degradation?

- To what extent are rigidity and flexibility of peptides manifested in the backbone and can these elements be exploited in designing more potent peptide analogues, or conversely, peptide antagonists? What are the related consequences of introducing peptide backbone modifications in terms of their altered electronic and stereochemical

properties, hydrophilicity and hydrophobicity, and their effects on adsorption, transport, and the ability to penetrate the blood-brain barrier?

Partial answers to these questions emerge. Work covered in Spatola's review, and on peptidomimetics in general since then, suggests that in the context of peptide hormone - receptor binding, the peptide backbone largely serves to position the essential side chains[16] (hence the advent of scaffold mimetics[8,16] and Hruby's topographical design approach[44,45]). In contrast, with protease inhibitors a non-scissile peptide bond surrogate (especially one that can mimic the transition state of amide bond hydrolysis) at the substrate cleavage site is a key approach to activity,[8,9,38,42] and enzyme - substrate / inhibitor binding involves hydrogen bonding to the substrate / inhibitor backbone peptide bonds.[16] Well-placed peptide bond surrogates do indeed increase the biological half-lives of their parent peptides: nearly all peptide bond surrogates, with the notable exception of the ester and thioester surrogates, are more stable to enzymatic hydrolysis than the natural peptide bond.[38,48] Most peptide bond surrogates do not greatly restrict global peptide conformation, but their different influences on the conformational preference of adjoining residues, and their capacity to form hydrogen bonded secondary structures, are much studied and frequently important. Alterations of electronic properties introduced by peptide bond surrogates can significantly affect the transport properties of their parent peptides.[49,50]

Thus the role of peptide bond surrogates is to increase bioavailability and (often subtly) to influence the conformation of the parent peptide; and more besides in the case of the protease inhibitors.

In addition, those peptide bond surrogates which mimic the transition-state of amide bond hydrolysis, or conversely the aminolysis of an amino acid, find use in haptens for the generation of catalytic antibodies.[51-53] Up until now, success in this field is largely restricted to the phosphorus based surrogates (see table 1),[51,52] *e.g.* in haptens used to raise antibodies which catalyse peptide bond formation.[54-56]

A few comparisons of peptide bond surrogates appear in the literature. In his review, Spatola compared peptide bond surrogates with respect to enzyme stability,

hydrophobicity, conformational influence, *etc*.[38] Fauchère and Thurieau reviewed the *in vivo* proteolytic degradation of endogenous peptides and the stabilising influence of (backbone) modifications used in (potentially) therapeutically useful modified peptides, in order to aid rational drug design.[48] Fincham *et al.* compared eleven peptide bond surrogates by modelling physiochemical properties (dimensions, volume, lipophilicity and hydrogen bonding capability) and biologically in the specific context of two cholecystokinin (CCK)-B ligands.[57] Most of their analogues displayed lower binding affinity than the parent ligands, but no correlation with the modelled properties was determined, suggesting that factors such as conformation are more important in this case. Nonetheless the study constitutes progress towards a rationale of peptide bond surrogate selection.[57]

Significant surrogates are presented in table 1, with leading references. Other surrogates exist, including some which constitute backbone extension, deletion, and cyclisation, and others which can only be used at peptide termini.[38]

Table 1:

Name	Symbol	Comments
N-Substituted	ψ(CONR)	R = Me, NH_2 or OH: conformational implications.[58,59]
Ester (depsipeptides)	ψ(CO_2)	Implications for turn formation: conformational study.[60]
Ketomethylene	ψ($COCH_2$)	Synthetic methodology for the core units: $R^1COCH_2CHR^2CO_2R^3$; R^1 = alkyl, heterocycle or ZNHCHBn; R^2 = H or alkyl; R^3 = H, alkyl, Pro-OMe or NHCHMeBn.[61,62]
Reduced or methyleneamino	ψ(CH_2NH)	Synthesis and conformational analysis of a cyclic Arg-Gly-Asp (RGD) pseudopeptide.[63]
Thioamide	ψ(CSNH)	Compatibility with reverse turn: conformational study.[64]

Phosphinate (n = 1) / phosphonate (n = 2) (and their esters)	ψ[$PO_{n+1}R(CH_2)_{2-n}$]	R = H; n = 1, 2: SPS methodologies for the incorporation of Pheψ(PO_2HCH_2)Gly,[65] Pheψ(PO_3H)Gly,[66] and Xaaψ(PO_3H)Leu.[67]
Phosphonamidate (and phosphonamidate ester)	ψ(PO_2RNH)	R = Me or H: HIV-1 protease inhibitor.[68]
Retro	ψ(NHCO)	Reviews.[69,70]
Alkene (*trans*)	ψ(*E*-CR=CH)	Synthetic methodology for Xaaψ(*E*-CH=CH)Yaa (R = H)[71] and Alaψ(*E*-CR=CH)Xaa (R = H or Me)[72] via S_N2'-attack of organocuprates on γ,δ-epimino-α,β-enoates.
	ψ(*E*-CMe=CMe)	β-Hairpin promoter.[73]
Fluoroalkene	ψ(*Z*-CF=CH)	Assessment of binding of tripeptide inhibitors to thermolysin.[74]
Carba or dimethylene	ψ(CH_2CH_2)	Synthesis, conformational study and SAR of a substance P NK-1 agonist.[75]
Thioether	ψ(CH_2S)	Review [including mention of sulfoxide variant, ψ(CH_2SO)].[76]
Hydroxyethylene	ψ[$CH(OH)CH_2$]	Synthetic methodology for the lactone derivative leading to Xaaψ[$CH(OH)CH_2$]Phe, from α-amino ketones.[77]
Dihydroxyethylene	ψ[CH(OH)CH(OH)]	Synthetic methodology for the lactone corresponding to PG-Leuψ[CH(OH)CH(OH)]Ala, from *N*-protected leucinal.[78,79]
Methylene-oxy	ψ(CH_2O)	Conformational implications.[80]
Tetrazole	ψ(CN_4)	*Cis* amide bond surrogate: synthesis and solution conformation of an active, cyclic somatostatin analogue.[81]

Cyanomethyleneamino	ψ[CH(CN)NH]	SPS of an NT(8-13) analogue with moderate binding affinity.[82,83]
Retrothioamide	ψ(NHCS)	Synthetic methodology for Xaaψ(NHCS)Yaa via endothionation of Xaaψ(NHCO)Yaa, and subsequent elongation.[84]
Retroreduced*	ψ($NHCH_2$)	Synthetic methodology for Ac-Pheψ(NHCO)Pheψ($NHCH_2$)-(*R,S*)Val-OR.[85]
Sulfonamido†	ψ(SO_2NH)	Synthesis of the glutathione disulfide analogue, [Glu-ψ(SO_2NH)Cys-Gly]$_2$.[86]
Sulfinamido (n = 1) / sulfonamido (n = 2)	ψ($CHRSO_nNH$)	Synthetic methodology for Glyψ($CHRSO_nNH$)Xaa (R = Me or Bn) and Yaaψ(CH_2SO_nNH)Zaa via α- and β-substituted sulfinyl chlorides respectively, and subsequent elongation.[87,88]
Retrosulfonamide	ψ($NHSO_2$)	Synthesis and crystal structure of Boc-Pro-Leuψ($NHSO_2$)Gly-NH_2.[89]

* Retroreduced pseudopeptides are intrinsically unstable unless the surrogate is preceded by a retro amide bond.

† Sulfonamido [and sulfinamido, ψ(SONH)] pseudopeptides are intrinsically unstable when preceded by -NHCHR- (*i.e.* when the surrogate is inserted in the peptide backbone),[86,87] hence the advent of the subsequent entries.

1.4 *Retro-inverso Pseudopeptides.-*

The peptide bond provides the peptide backbone with a "sense of direction". Thus peptides and proteins are inherently non-palindromic. The convention for the construction of peptide names and sequence representation proceeds from the amino terminus (written on the left) to the carboxy terminus (written on the right).[2] Therefore the direction of the peptide bond is defined as that from the carbonyl carbon atom to the adjoining nitrogen atom.

Hence it is possible to envisage the retro-isomer of a peptide, *i.e.* an isomer in which the direction of the amino acid sequence is reversed;[90] see figure 5 (a) and (d) for a cyclic example, and figure 6 (a) and (d) for a linear example.

1.4.1 *Cyclic peptides.*

This concept of retro-isomers was recognised early in the study of the biological activity of peptides and was applied to cyclic peptides and cyclic depsipeptides (for an overview of this early work see reference 90). Shemyakin, Ovchinnikov and co-workers,[91,92] and later Wieland and co-workers,[93] further recognised that reversal of the residue sequence of the enantiomer of a cyclic (depsi)peptide (or conversely, enantiomerisation of the retro-isomer) maintains topochemical complimentarity between the parent cyclic (depsi)peptide and its isomer.[90] The resultant isomer is called a cycloretro-enantiomer (or, for the isomer of an all L-cyclopeptide, a retro-all-D-cyclopeptide[93]) and is defined as an isomer of a cyclic compound in which the sequence is reversed and each residue is inverted,[90] figure 5.

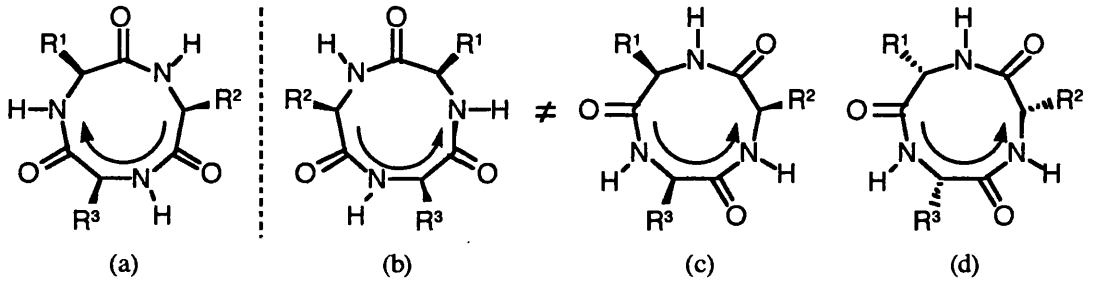

Figure 5: Cyclo(-Xaa-Yaa-Zaa-) and some of its cycloisomers; (a) cyclo(-Xaa-Yaa-Zaa-); (b) the enantiomer, cyclo(-D-Xaa-D-Yaa-D-Zaa-); (c) the cycloretro-enantiomer, cyclo(-D-Zaa-D-Yaa-D-Xaa-); and (d) the retro-isomer, cyclo(-Zaa-Yaa-Xaa-); where R^1 is the side chain of the amino acid Xaa, *etc.*

It is important to note that: (1) the cycloretro-enantiomer is not the same isomer as the enantiomer of a cyclic compound, *c.f.* figure 5 (c) and (b); and (2) there are (many) other cycloisomers of cyclic peptides (see reference 90 for further discussion).

Shemyakin, Ovchinnikov and co-workers synthesised the cycloretro-enantiomer of [Gly^5,Gly^{10}]gramicidin S, which displayed similar antimicrobial activity to that of the parent [Gly^5,Gly^{10}]gramicidin S.[91,92,94]

1.4.2 *Linear peptides.*

The extension of the retro-enantiomer concept to linear peptides was hampered by their possession of end groups,[90] which are obviously absent in cyclic peptides. Thus reversion of sequence and enantiomerisation of a linear peptide produces an isomer topochemically complimentary to the parent peptide along the chain length, but with non-complimentary end groups: figure 6.[90]

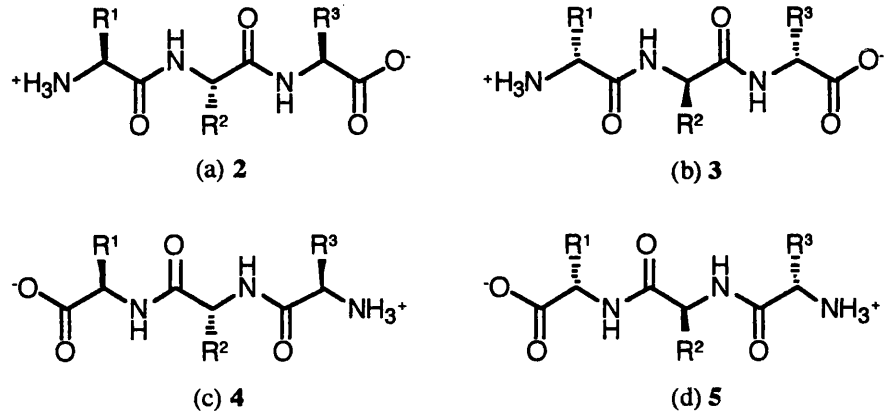

Figure 6: (a) Xaa-Yaa-Zaa, **2**; (b) the enantiomer, D-Xaa-D-Yaa-D-Zaa, **3**; (c) the retro-inverso peptide, D-Zaa-D-Yaa-D-Xaa, **4**, obtained from **3** by reversal of sequence, or from **5** by enantiomerisation; and (d) the retro-isomer, Zaa-Yaa-Xaa, **5**.

The term retro-inverso peptides (or retro-inverso pseudopeptides, the "pseudo" being tautologous in this context) is used to describe the relationship between peptides **2** and **4**, (and that between **3** and **5**) because **4** is obtained from **2** (and *vice versa*) by reversal of the direction of the amino acid sequence and inversion of each chiral centre.[90,‡] The term retro-inverso peptide is preferable to retro-enantio peptide in this linear peptide context, because the latter implies too much structural equivalence between the parent peptide, **2** and the isomer, **4**.[90]

Although Shemyakinin and co-workers synthesised an effective retro-inverso pepsin inhibitor without complimentary end groups,[92] the non-complimentarity of end groups (termed the "end group problem") was recognised by the pioneers of the retro-

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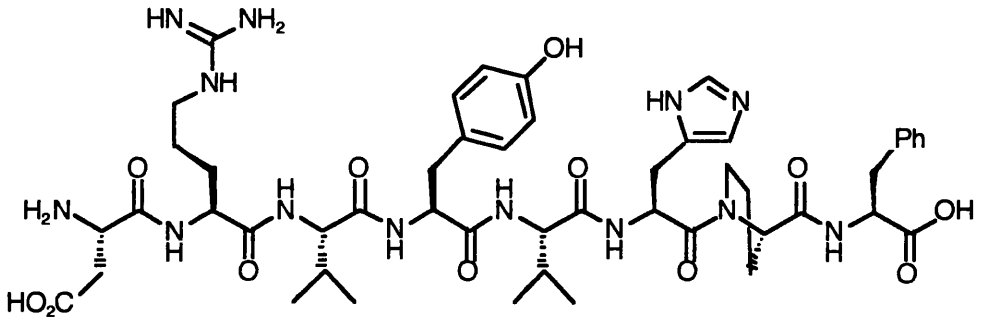
‡ Note that for the two coded α-amino acids with chiral C^{β}s, namely threonine and isoleucine, enantiomerisation results in topochemical noncomplimentarity with the parent peptide at C^{β}. This is often ignored in retro-inverso studies, but may be overcome by incorporation the appropriate D-allo-amino acid, or by suitable choice of synthetic method for *g*Xaa residues [see section **1.5.1***(b)(ii)*].

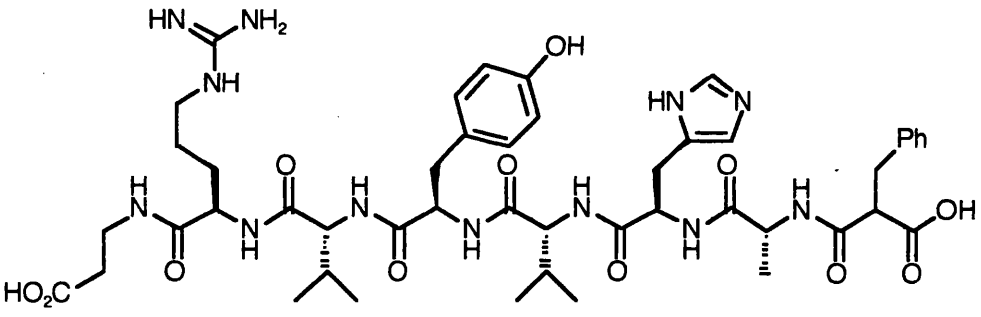
inverso concept as the probable cause of the inactivity of various retro-inverso peptide hormones synthesised by early researchers.[90,92,§]

(a) End group modification

These set-backs prompted the development of end group modifications, designed to improve complimentarity between the parent peptide and its retro-inverso isomer at the chain termini. Various modifications were employed with differing degrees of success (as judged by the retention of biological activity by the end group modified retro-inverso peptides).[90,92] Paiva and co-workers synthesised the first biologically active end group modified retro-inverso peptide hormones, two angiotensin analogues, which demonstrated that the (C^2 substituted) malonyl (or malonamyl) residue suggested by Rudinger[96] was suitable for carboxy terminal modification: figure 7.[97]



(a)



(b)

Figure 7: (a) angiotensin and (b) end group modified retro-inverso-[Ala^7]-desamino-angiotensin.

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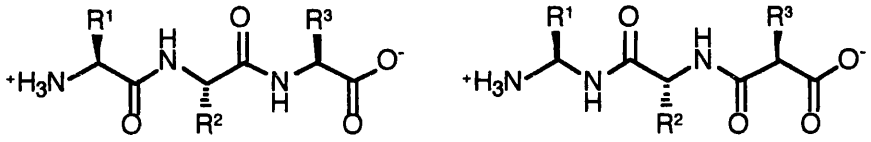
§ However, the inactivity of those retro-inverso peptide hormones containing proline, *e.g.* bradykinin, could equally well be due to the lack of spatial coincidence between the proline rings of, *e.g.* bradykinin and retro-inverso bradykinin. This topological difference was dubbed the "proline problem", and arises because the proline side chain is cyclised onto the proline backbone nitrogen, which changes position with the carbonyl carbon in the retro-inverso isomer.[90,92,95]

Hayward and Morley first proposed (and incorporated) the *gem*-diaminoalkyl residue as an amino terminus modification.[98] They replaced a pyroglutamyl residue with a 5-oxo-2-pyrrolidinylamino residue in a model pseudodipeptide, **48** (see scheme 16),[98] but did not follow up this work. As shown in figure 8, the incorporation of an amino terminal *gem*-diaminoalkyl residue and a carboxy terminal (C^2 substituted) malonyl residue results in good structural complimentarity between the parent peptide, **2**, and its end group modified retro-inverso isomer, **6**.



(a) **2** (b) **6**

Figure 8: (a) Xaa-Yaa-Zaa, **2**; and (b) the end group modified retro-inverso isomer, Xaaψ(NHCO)Yaaψ(NHCO)Zaa, **6**.

Chorev and Goodman took up the retro-inverso concept and, through the general synthetic methodology they established, are responsible for the widespread adoption of the *gem*-diaminoalkyl residue as an amino terminus modification.[90,99]

1.5 *Partially Modified Retro-inverso Peptides: the Retro Peptide Bond as a True Peptide Bond Surrogate.-*

Gem-diaminoalkyl and C^2 substituted malonyl residues are not restricted to terminal incorporation. When these residues are incorporated within the peptide sequence, a new class of peptide isomers are produced. Hayward and Morley first introduced this concept,[98] but it was Chorev and Goodman who first implemented it and who termed the resultant isomers partially modified retro-inverso (PMRI) peptides.[90] Such isomers consist of the parent peptide sequence with two (or more) internal residues replaced by *gem*-diaminoalkyl and C^2 substituted malonyl residues, and with the intervening amino acids having inverted configuration and reversed sense of direction; *i.e.* an end group modified retro-inverso sequence is incorporated within a peptide sequence: figure 9.[90,98] [Note that an end group modified retro-inverso isomer, such as that depicted in figure 8 (b), is simply a special example of a PMRI peptide with <u>all</u> the amide bonds reversed and amino acids inverted. Therefore all subsequent discussion of PMRI

peptides also applies to this special case]. The application of the *gem*-diaminoalkyl and C^2 substituted malonyl residues in this context thus makes the retro peptide bond into a true peptide bond surrogate, in that any <u>one</u> peptide bond can be replaced; see figure 9 (d) where only the peptide bond between residues 5 and 6 is reversed.

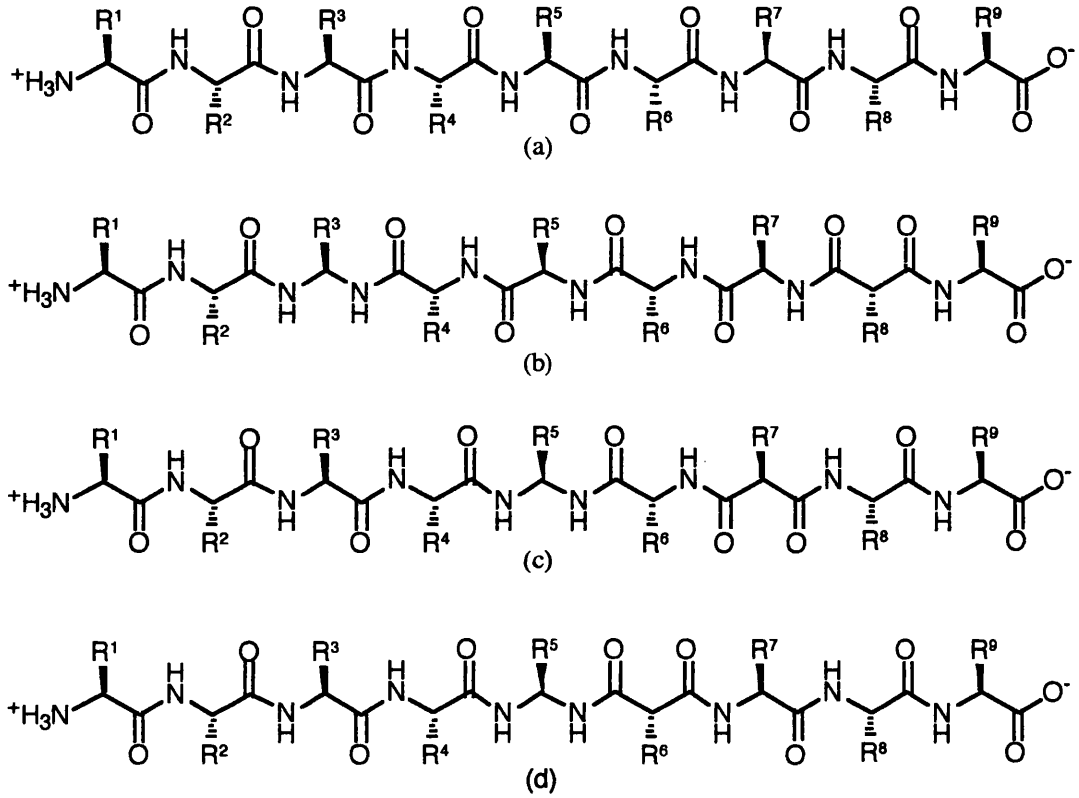


Figure 9: (a) The parent peptide and its PMRI isomers, (b) 5 bonds reversed, (c) 2 bonds reversed, and (d) 1 bond reversed.¶

Chorev and Goodman exhaustively reviewed the field of PMRI peptides in 1993[69] and further reviewed developments in enantio-, retro-, and retro-inverso peptides and proteins in 1995.[70] Therefore subsequent discussion here is largely restricted to PMRI peptides, taking pertinent examples from the literature, with special note of material not covered by Chorev and Goodman.

1.5.1 *Synthetic methodology.*

The principal concern of PMRI peptide synthesis is the construction and coupling of the *gem*-diaminoalkyl and C^2 substituted malonyl residues, on which this section

¶ Figure 9 (b) and (c) are examples of structures that are more conveniently represented using the *g* / *m* / *r* system of symbols, rather than the ψ system.

focuses. Assembly of the remainder of the PMRI peptide chain (merely) involves the protection and coupling of L- or D-α-amino acids, which is the (by no means trivial) stuff of standard peptide chemistry.[100,101] As with standard peptide chemistry the methodology is readily divided into two categories: that carried out (a) in solution, and (b) on a solid support (SPS); there being considerable common ground between the two.

(a) Solution phase methodology

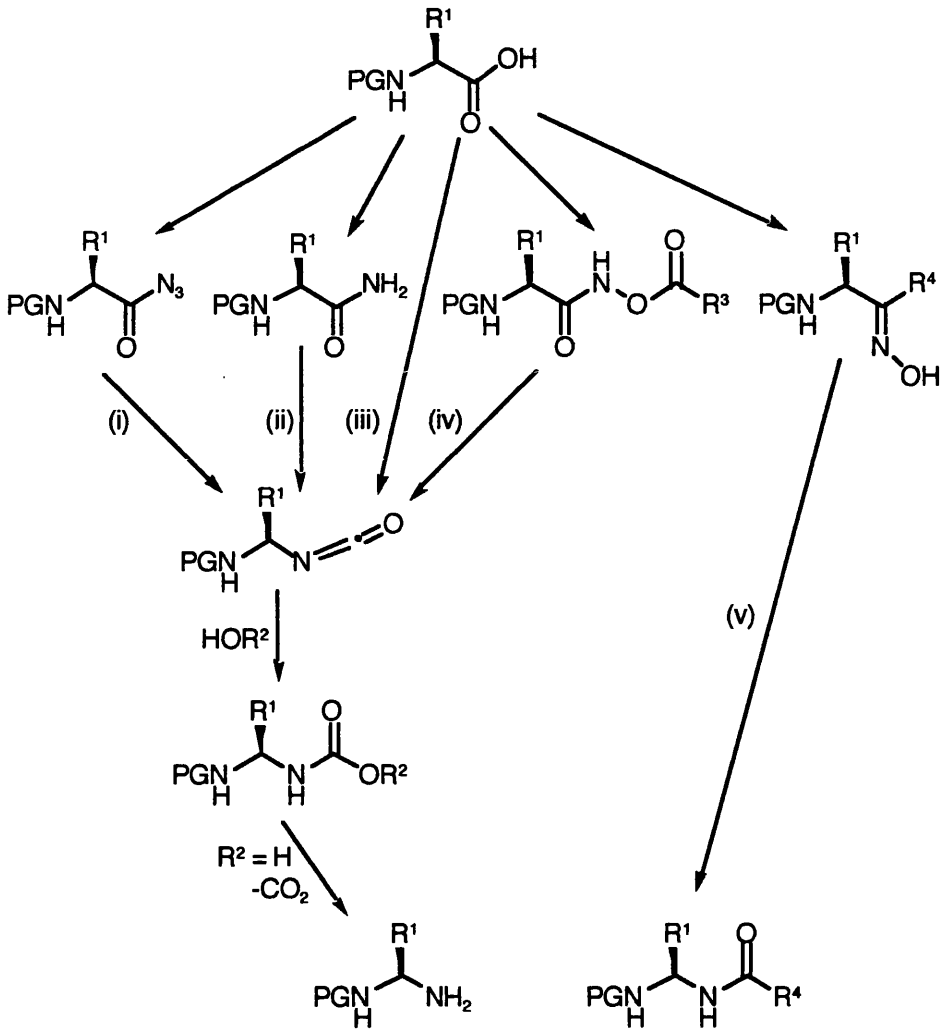
(i) Gem-*diaminoalkyl derivatives*

(1) Rearrangements

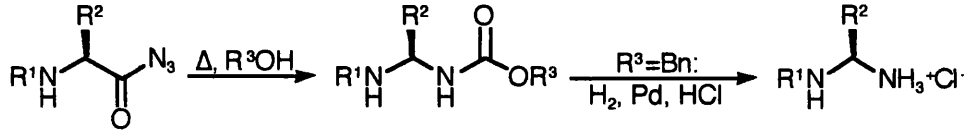
Most of the pioneering syntheses of *gem*-diaminoalkyl derivatives for PMRI peptides used a rearrangement step to obtain the *gem* relationship of the amino groups. Rearrangements remain the most common methods used for this manipulation. In theory, any of the rearrangements depicted in scheme 1 could be employed, but in practice only the Curtius and Hofmann rearrangements (in various forms) are utilised in this context.

During all these rearrangements the migrating group ($PGNHCHR^1$ in scheme 1) retains its configuration; thus an L-α-amino acid as starting material yields the topographically complimentary *gem*-diaminoalkyl derivative.

The Curtius rearrangement was employed by Chorev, Goodman and co-workers in their first preparations of PMRI peptides.[90,99,102] Their approach to the *gem*-diaminoalkyl residues, **8** and **9**, was based on the Bergmann and Zervas stepwise degradation method ("carbobenzoxy degradation") for polypeptide sequencing: scheme 2.[103] But, rather than degrade the *gem*-diaminoalkyl derivatives, **8** and **9**, to the corresponding aldehydes, **10**, for identification, Chorev, Goodman and co-workers utilised them in their PMRI peptides.[90,99,102] As indicated in scheme 2, they applied this methodology to both protected peptides, **7(a)**, leaving the *gem*-diaminoalkyl group attached to the peptide chain [**8(a)**] and to *N*-protected amino acids, **7(b)**, leading to isolated, orthogonally protected (when R^1 and R^3 in scheme 2 are complimentary) *gem*-diaminoalkyl derivatives [**8(b)**].[90,99,102] This latter synthesis obviously requires an additional deprotection and coupling step over the former, in order to assemble the PMRI peptide.



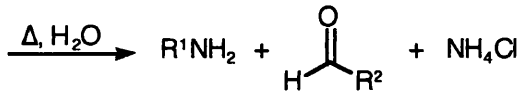
Scheme 1: Rearrangements leading to *gem*-diamino derivatives. (i) Curtius: Δ. (ii) Hofmann: NaOBr. (iii) Schmidt: HN_3. (iv) Lossen: OH^-. (v) Beckmann: PCl_5.



7
(a): R1=peptide chain
(b): R1=PG

8

9



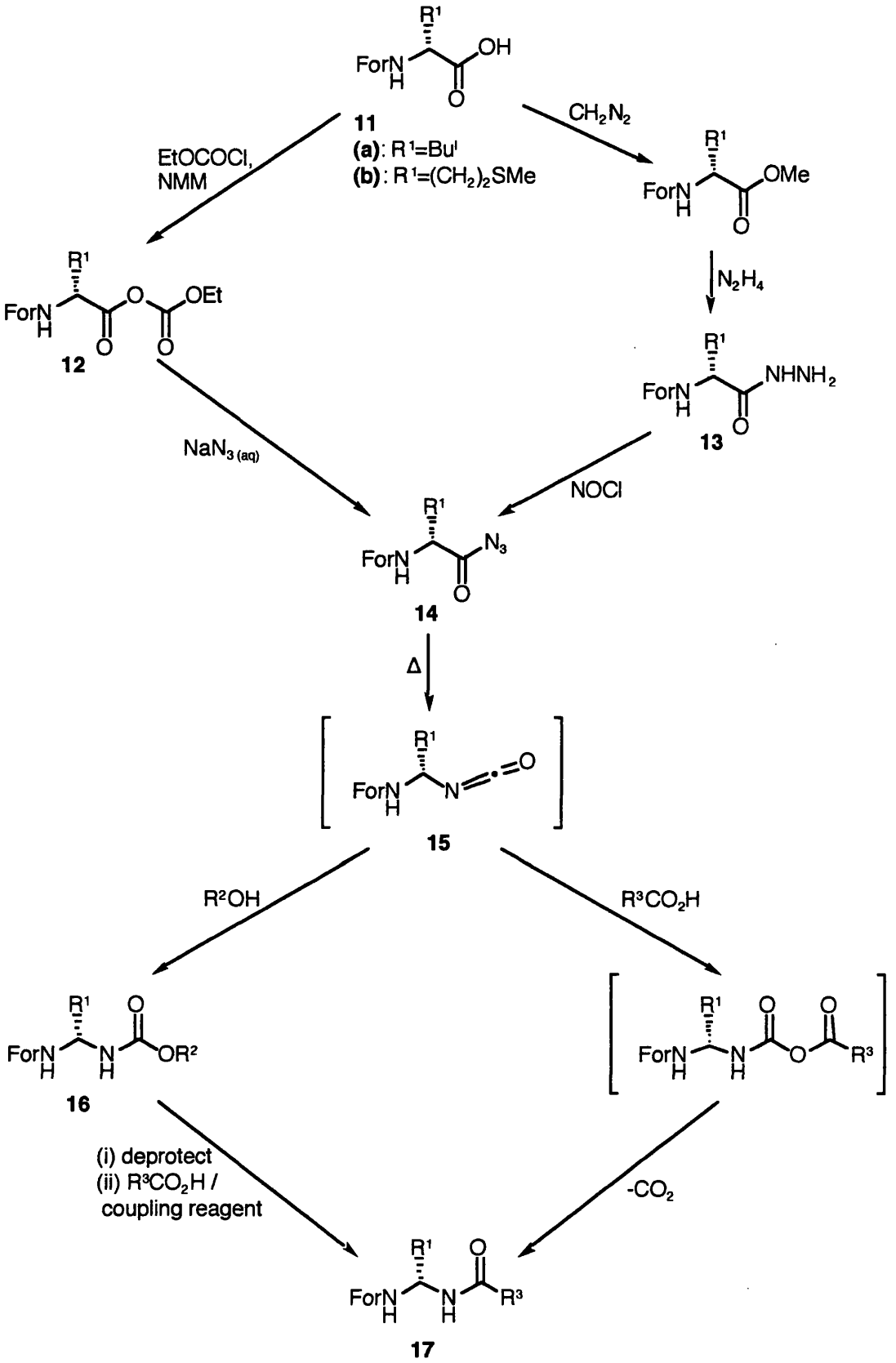
10

Scheme 2: Carbobenzoxy degradation (R^1 = peptide chain and R^3 = Bn) via *gem*-diaminoalkyl derivatives, **8(a)** and **9(a)**.

Of course, this approach raises its own questions: (1) how best to synthesise the required α-aminoacyl azides, **7**? (2) which are the best protecting groups (R^1 and CO_2R^3

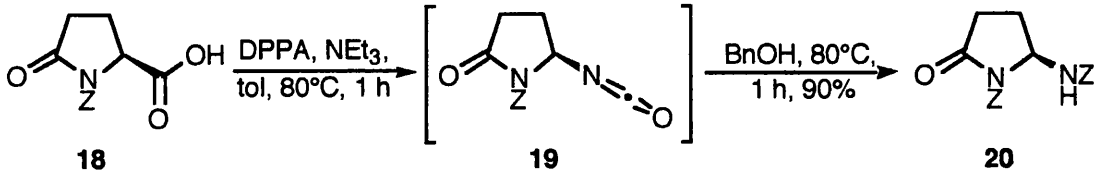
in scheme 2) for the *gem*-diaminoalkyl derivatives, **8**? (3) which is the best coupling procedure to employ with the *gem*-diaminoalkyl derivatives, **9**? and (4) is it better to synthesise free or peptide bound *gem*-diaminoalkyl derivatives, *i.e.* **8** and **9 (a)** vs **(b)**?

Chorev, Goodman and co-workers performed limited comparative studies on the use of the Curtius rearrangement, in order to begin to answer these questions.[99,104,105] Since acyl azides have been heavily utilised in peptide chemistry as acylating agents[100,106] (which probably explains their immediate popularity for PMRI peptide synthesis), a number of syntheses of α-aminoacyl azides, **7**, exist.[100,106] Chorev and Goodman compared two methods of *N*-formylaminoacyl azide, **14** preparation: nitrosylation of an *N*-formylaminoacyl hydrazide, **13**, and formation of a mixed anhydride, **12**, followed by nucleophilic displacement by sodium azide: scheme 3.[104] Curtius rearrangement of the α-aminoacyl azides, **14**, yielded the isocyanates, **15**, which were trapped with benzyl or *tert*-butyl alcohol to yield the orthogonally protected *gem*-diaminoalkyl residues, **16**, or reacted with a carboxylic acid derivative to yield a PMRI peptidic unit, **17**, directly (the so-called "Goldschmidt and Wick type reaction", after the chemists who employed a closely related procedure for peptide synthesis[107,108]).[104] Chorev and Goodman concluded that the mixed anhydride procedure results in better yields of the desired *gem*-diaminoalkyl derivatives, **16**, than the nitrosylation method.[104] However, they did not test the *tert*-butyl nitrite variant of the nitrosylation method. Moutevelis-Minakakis and Photaki[109] used this method and obtained yields of orthogonally protected *gem*-diaminoalkyl derivatives comparable with those of Chorev and Goodman's nitrosyl chloride method[104] (11-45%); therefore, in this context, nitrosylation is generally inferior to the mixed anhydride method of α-aminoacyl azide preparation.



Scheme 3: Chorev and Goodman's comparative Curtius rearrangements. Yields: **16(a)** (R^2 = Bn), 66 and 77%; **16(b)** (R^2 = Bu^t), 37 and 66%; for the NOCl and NaN_3 routes respectively (from **11**). $R^3 = R^4NHCHBn$; **(a)** R^4 = Z, **(b)** R^4 = Boc.[104]

The other popular method for the synthesis of α-aminoacyl azides *en route* to PMRI peptides uses the reagent diphenylphosphoryl azide (DPPA).[110,111] Reaction of DPPA with an *N*-protected α-amino acid (*e.g.* **18**) or peptide, yields the corresponding acyl azide (which is not isolated). Under the usual reaction conditions the intermediate acyl azide undergoes the Curtius rearrangement to yield the corresponding isocyanate (*e.g.* **19**), which is trapped with an appropriate alcohol (either *in situ* or added subsequently) to furnish the desired *gem*-diaminoalkyl derivative (*e.g.* **20**): scheme 4.[90,109,112]



18 **19** **20**

Scheme 4: An example of the use of DPPA for the synthesis of the protected *gem*-diaminoalkyl derivative, Z-*g*Glp-Z, **20**.[90,112]

DPPA may be used quite generally for the synthesis of *gem*-diaminoalkyl derivatives, without the need for extraordinary side chain protection.[113] However, a "one-pot" Goldschmidt and Wick type reaction is, of course, not possible without ensuring complete consumption of the DPPA prior to addition of the second carboxylic acid.

No direct comparison of the DPPA method with other approaches to α-aminoacyl azide synthesis has been undertaken, nor have variants of this reagent[114] found widespread application in PMRI peptide synthesis (but see references 115 and 116).

The literature contains other classical and more modern routes to acyl azides;[117-120] these have been but little used in PMRI peptide synthesis. The only example of the use of TMS-azide to synthesise an acyl azide *en route* to a PMRI peptide is Fincham *et al.*'s synthesis of CCK-B ligands.[57] They synthesised 2-Adoc-Trp-N_3 by reaction of the amino acid mixed anhydride and TMS-azide. Curtius rearrangement and trapping of the isocyanate with *p*-nitrobenzyl alcohol / DABCO furnished racemic 2-Adoc-(*R*,*S*)*g*Trp-OBn(*p*-NO_2) in 42% yield.[57] They offered no explanation for the racemisation.

The criteria against which to judge protecting groups for the *gem*-diaminoalkyl derivatives are, as with any protecting groups, the efficacy of protection [indicated by the

prevention of decomposition and side-reactions (including racemisation)] and their ease of incorporation and removal.

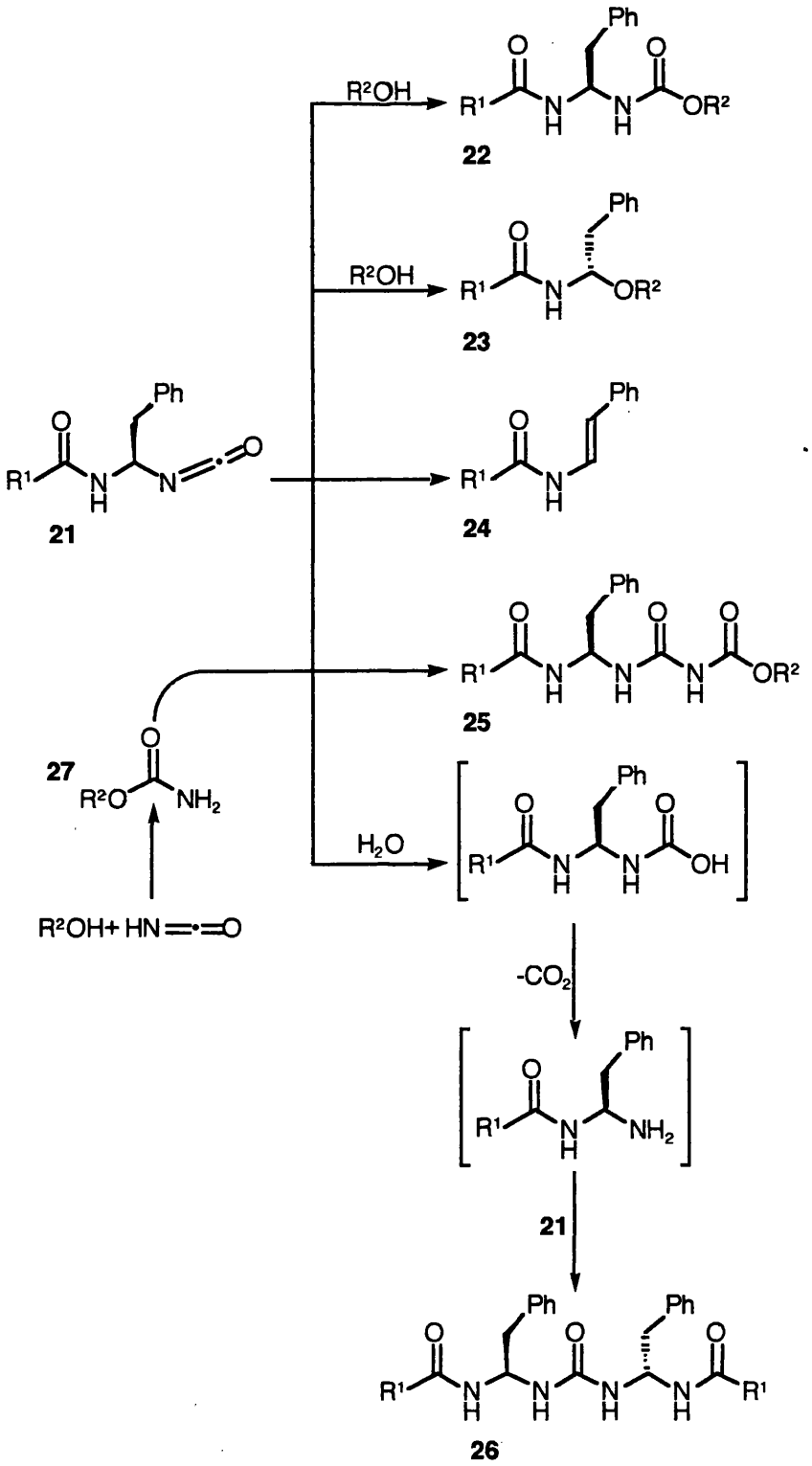
Chorev, Goodman and MacDonald studied side reactions during the synthesis of protected *gem*-diaminoalkyl derivatives obtained by the Curtius rearrangement of acetyl, Boc or Z-protected phenylalanyl azide, trapping the intermediate isocyanate *in situ* with methanol or benzyl alcohol.[105] They obtained the desired *gem*-diaminoalkyl derivatives, **22**, plus numerous by-products, in a ratio that depended upon the nature of the phenylalanyl azide *N*-protecting group, and the nature and molar excess of the alcohol.[105] They rationalised the formation of the by-products as shown in scheme 5; the yields are shown in table 2.

Table 2:

Isocyanate	R^1	Alcohol, eq*	Product, yield† / %				
			22	**23**	**24**	**25**	**26**
21(a)	BnO	MeOH, 20	49	9	6	5	1
		MeOH, 2	65	nr	nr	2	nr
21(b)	Bu^tO	MeOH, 20	29	44	nr	12	nr
		MeOH, 2	45	nr	nr	4	nr
		BnOH, 10	18	18	nr	20	nr
		BnOH, 2	31	nr	nr	18	nr
21(c)	Me	MeOH, 10	78	2	nr	nr	nr

* They rationalised the increase in the yield of by-products on increasing the molar excess of alcohol (a finding contrary to their original expectation) by reasoning that increasing the alcohol content leads to an increasingly polar reaction mixture (the solvent being toluene) which relatively favours the heterolytic or displacement reactions. A control experiment proved that the desired product, **22(b)** (R^2 = Me), is stable under the reaction conditions.[105]

† Isolated yields relative to starting *N*-protected phenylalanine.



Scheme 5: Formation of by-products during the synthesis of bis-protected-1,1-diamino-2-phenylethane, **22**, by the addition of alcohol to the isocyanate, **21** (no racemisation was observed). The 1-amino-1-alkoxy-2-phenylethane by-product, **23**, arises from cyanate displacement by the alcohol; whereas the styrene by-product, **24**, arises by elimination of cyanate. Reaction of displaced cyanate and alcohol produces protected ammonia, **27**, which adds to the isocyanate, **21**, to yield the allophanate by-product, **25**. Traces of water present in the reaction mixture lead to the formation of the urea by-product, **26**.[105]

As regards the choice of protecting group for *gem*-diaminoalkyl derivatives, they concluded that acetyl is better than Z, which is better than Boc, on the basis of maximisation of product yield and concurrent minimisation of the yield of by-products.[105] They reasoned that acetyl protection resulted in fewer by-products than carbamate protection due to a reduced tendency of isocyanate **21(c)** to undergo the heterolytic or displacement reactions.[105] Although acetyl is not a practical amino protecting group, they argued that it is a suitable model for a peptide chain, and therefore the results they obtained suggest that, in answer to question (4) above, it is better to synthesise peptidyl *gem*-diaminoalkyl derivatives.[105]

DeBons and Loudon and Moutevelis-Minakakis and Photaki both obtained urea by-products, corresponding to **26**, in their studies of *gem*-diaminoalkyl derivative synthesis.[109,115] Both argued that the water required for urea formation is generated *in situ* by the dehydration of *tert*-butanol by the intermediate isocyanate.[109,115] This provides a basis to disfavour *tert*-butanol as an isocyanate trap. Chorev, Goodman and Willson[99] counselled against Boc deprotection of *gem*-diaminoalkyl derivatives when hydrogenation of a Z group is possible instead, but offered no rationale. Previous work in this department on the synthesis of PMRI dipeptides using the Goldschmidt and Wick type reaction demonstrated that Boc and Z groups are equally effective as protecting groups during the synthesis, but that Boc deprotection by conventional acid hydrolysis results in decomposition.[84]

So, we may conclude from these results that Z is to be favoured over Boc for the protection of *gem*-diaminoalkyl compounds.‡

Fmoc and 2-nitrophenylsulfenyl are also acceptable protecting groups for *gem*-diaminoalkyl compounds, and provide additional, orthogonal dimensions;[121] no comparison of these with the other protecting groups discussed has been undertaken.

Regarding the coupling of *gem*-diaminoalkyl derivatives to carboxylic acids [question (3) above], no comprehensive comparison of the vast array of available methods

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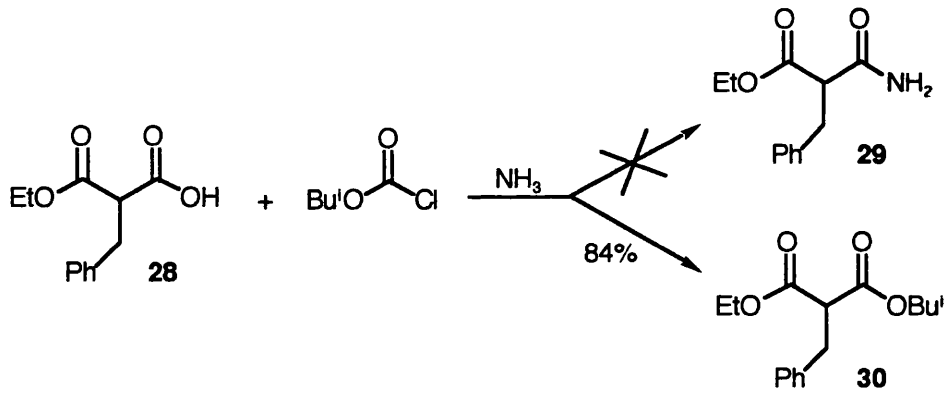
‡ Loudon and co-workers studied the hydrolysis of *gem*-diamino alkyl derivatives. Their results, which are summarised below [section **1.5.1***(a)(i)(5)*], have similar implications for the choice of *gem*-diamino alkyl derivative protecting groups.

has been undertaken (in theory, any peptide coupling procedure[100,122] is applicable). Due to the danger of *gem*-diaminoalkyl derivative decomposition [see section **1.5.1***(a)(i)(5)*], a rapid coupling procedure is essential; however, most peptide coupling procedures currently in use meet this requirement. The method used almost exclusively by researchers engaged in PMRI peptide synthesis is the *N*-hydroxybenzotriazole (HOBt) or *N*-hydroxysuccinimide (HOSu) catalysed carbodiimide (usually DCC) procedure (however the BOP reagent has also been successfully employed[123,124]). Although catalysed carbodiimide mediated couplings give good results, activated malonates have a lower coupling efficiency than normal amino acids.[121] Thus yields tend to be lower, and reaction times longer than those usually employed.[121] In addition, couplings to *gem*-diaminoalkyl derivatives tend to be sluggish *per se*.[121] The reduced coupling efficiency of both modified residues is presumably electronic in origin.[125] Therefore, a wise strategy for the assembly of a PMRI peptide avoids fragment couplings (*i.e.* the coupling of partial sequences, as opposed to individual residues) to *gem*-diaminoalkyl derivatives, if possible.[121]

The only coupling procedure found to be unsatisfactory is the mixed anhydride method. Goodman and co-workers discovered that the mixed anhydride coupling of monoethyl 2-benzylmalonate, **28**, to ammonia, yielded only ethyl *iso*-butyl 2-benzylmalonate, **30**, and none of the expected amide, **29**: scheme 6.[121]



Scheme 6: A failed mixed anhydride coupling.

The Goldschmidt and Wick type reaction obviates the need for such a coupling step altogether when the *gem*-diaminoalkyl group is formed at the peptide carboxy-terminus; or reduces the number of necessary couplings from two to one for free *gem*-

diaminoalkyl groups. Chorev and Goodman, in their aforementioned comparison of Curtius rearrangements,[104] found that the yield of the Goldschmidt and Wick type reaction varied with the carboxylic acid (*e.g.* R^3CO_2H, scheme 3) employed. *N*-Protected phenylalanine (1.5 eq) gave a reduced yield of the PMRI dipeptide [**17(a)**, 35%; **17(b)**, 6%; scheme 3], but malonic acid (3 eq) gave an increased yield of PMRI tripeptide Boc-Phe-Alaψ(NHCO)Gly (47%) [changes relative to the corresponding multi-step deprotection and coupling procedures: **17(a)**, 66%; **17(b)**, 22%; and Boc-Phe-Alaψ(NHCO)Gly, 11%].[104]

The Goldschmidt and Wick type reaction, using a malonate monoester, was successfully used in this department to synthesise PMRI dipeptides, which are not readily prepared by acylation of mono-protected *gem*-diaminoalkyl compounds because of their tendency to decompose when the protecting group is a carbamate [see section **1.5.1***(a)(i)(5)*].[84,§]

Thus the Curtius rearrangement, with suitable precautions, provides ready access to *gem*-diaminoalkyl compounds appropriate for PMRI peptide synthesis and, in combination with the Goldschmidt and Wick type reaction is a valuable method for the direct synthesis of PMRI peptides.

The Hofmann rearrangement is extensively employed for the synthesis of PMRI peptides, exclusively using the mild oxidant iodobenzene bis(trifluoroacetate) (IBTFA);[127-129,¶] other conditions being too harsh.

IBTFA effects the Hofmann rearrangement on primary amides, under acidic or neutral conditions (giving an amine trifluoroacetate salt, **33**, and carbon dioxide), but does not affect secondary or tertiary amides.[127] Therefore IBTFA may be employed to synthesise mono-protected *gem*-diaminoalkyl derivatives from *N*-protected α-amino acid

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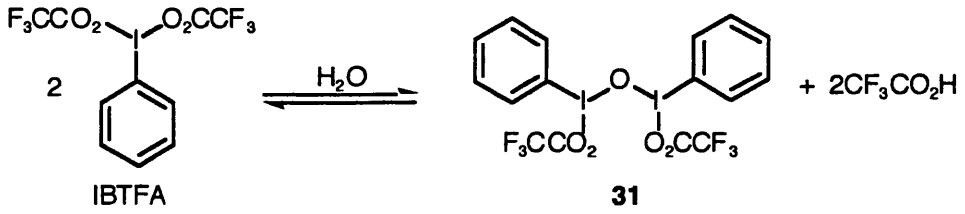
§ However, Verdini and co-workers have developed a non-carbamate nitrogen protecting group, specifically to stabilise monoprotected *gem*-diamino alkyl compounds, in order to synthesise PMRI dipeptides for use in SPS;[126] see section **1.5.1***(b)(ii)*.

¶ IBTFA is also known as phenyl iodosyl bis(trifluoroacetate) (PIFA or PIT), [bis(trifluoroacetoxy)iodo]benzene (TIB), phenylbis(trifluoroacetato-*O*)-iodine and iodoso benzene 1,1-bis(trifluoroacetate)!

primary amides or peptidyl *gem*-diaminoalkyl derivatives from peptidyl primary amides [which Loudon and co-workers hydrolysed to the corresponding aldehyde and a new peptidyl amide in their *C*-terminal sequential degradation process,[130] analogous to that of Bergmann and Zervas (scheme 2)].[121,127,131] However, when the α-amino acid *N*-protecting group is a carbamate, the resultant mono-protected *gem*-diaminoalkyl derivative decomposes under the reaction conditions.[121] Therefore, the requirement to favour the synthesis of peptidyl *gem*-diaminoalkyl derivatives encountered with the Curtius rearrangement [in answer to question (4), above] is absolute in the case of the IBTFA mediated Hofmann rearrangement.*

The mechanism of the IBTFA mediated Hofmann rearrangement is complex. Boutin and Loudon performed a mechanistic study (using hexanamide as substrate),[128] the results of which are in disagreement with the earlier study of Swaminathan and Venkatasubramanian.[132,†] Boutin and Loudon demonstrated that IBTFA forms a dimer, **31**, under the reaction conditions [water / acetonitrile (50:50)]: scheme 7.



Scheme 7: Dimerisation of IBTFA.

It is not clear, however, whether the dimer, **31**, or / and the IBTFA monomer is the reactive species; scheme 8 shows the mechanism, determined by Boutin and Loudon, with the dimer, **31**, as the reactive species.[128] The rate-determining step is the rearrangement of the amide - IBTFA dimer complex, **32**.[128]

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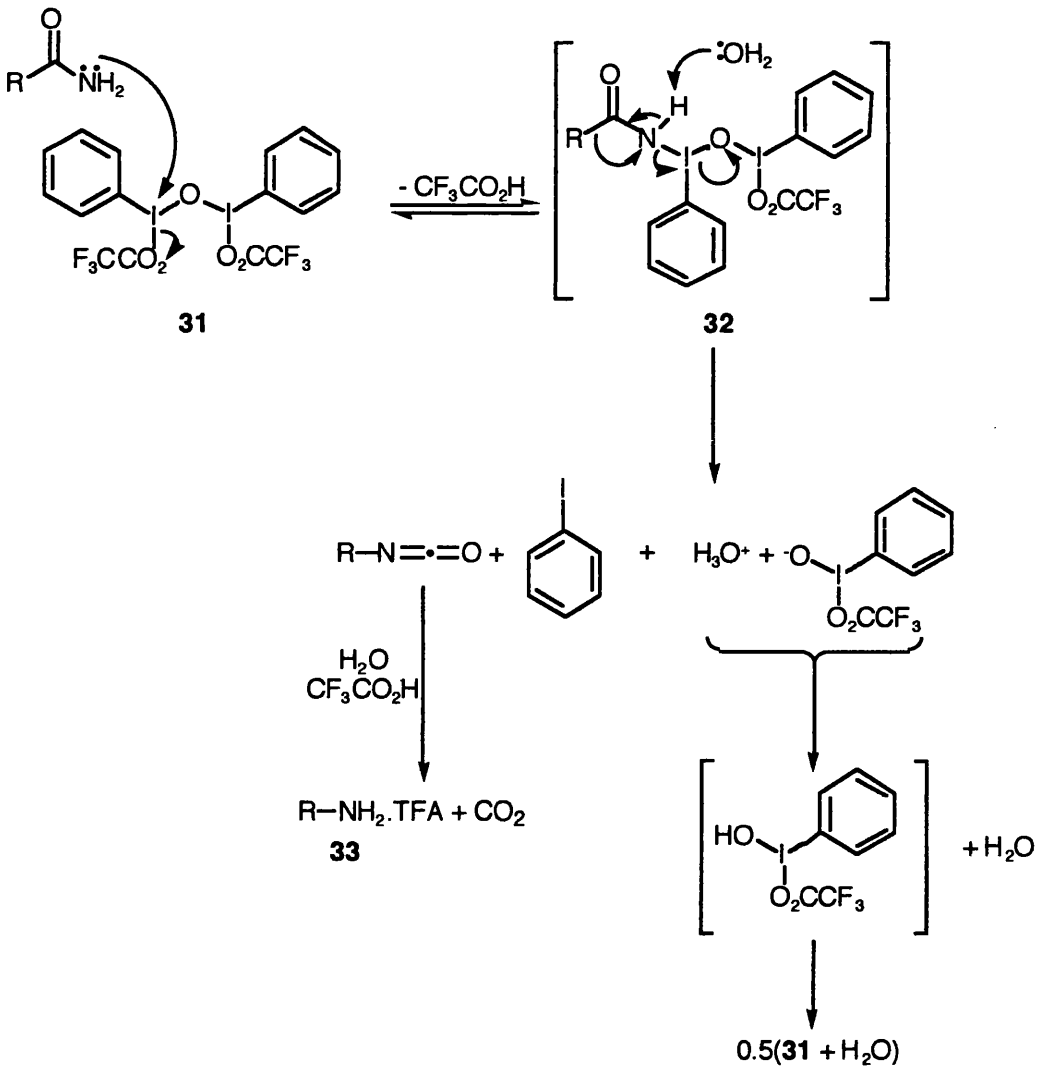
* Unless MNP protection is used: see section **1.5.1*(b)(ii)***.

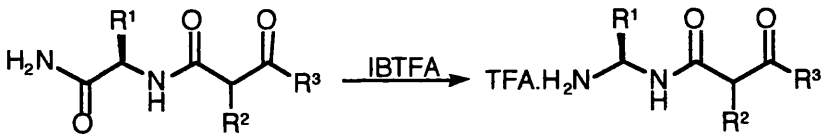
† Although this earlier study investigated the mechanism of action of iodobenzene diacetate, rather than IBTFA, with aromatic amide substrates.



Scheme 8: The mechanism of the IBTFA mediated Hofmann rearrangement.[128]

As this mechanism suggests, the reaction proceeds with complete retention of configuration of the migrating group.[127,131]

The considerations discussed above [question (3)] with respect to coupling procedures, also apply to the IBTFA procedure, although no Goldschmidt and Wick type reaction is known using IBTFA because the reaction conditions do not permit trapping, rather than hydrolysis, of the intermediate isocyanate. However, a conceptually analogous, direct synthesis of a PMRI dipeptide unit, **35**, is possible by the reaction of IBTFA with a malonylaminoacyl amide, **34**: scheme 9.[69,121]



34 35

Scheme 9: Direct synthesis of a PMRI dipeptide unit, **35**, using IBTFA. R^3 = peptide chain or OR^4, R^4 = alkyl, Bn.

Special considerations are necessary for the synthesis of Gln or Asn containing PMRI peptides using IBTFA. The side chains of these amino acids contain primary amides, which undergo the Hofmann rearrangement under the action of IBTFA.[130] Dürr, Goodman and Jung circumvented this problem by carrying out the IBTFA mediated Hofmann rearrangement on a protected Glu residue, which was subsequently converted to the required Gln by treatment with ammonia: scheme 10.[133] Their approach is generally applicable for the synthesis of PMRI peptides containing Asn and / or Gln, with appropriate protecting group manipulation.

The problems associated with the synthesis of *g*Asn are not confined to IBTFA. Cushman and co-workers were unable to produce satisfactorily protected *g*Asn from Z-Asn via the Curtius rearrangement using either the mixed anhydride or DPPA method of acyl azide synthesis.[123] They apportioned blame on the side chain amide group of Asn, which they therefore "protected" as a nitrile during synthesis: scheme 11.[123]

The oxidation sensitive amino acids, tyrosine, tryptophan and methionine also require special attention if they are to be exposed to IBTFA. Their side chains may be effectively protected with Bu^t, For and as Met(O), respectively.[69,121] IBTFA oxidises cysteine to cystine and cleaves or oxidises the usual cysteine protecting groups: no way around this problem exists, other than strategic assembly.[126]

No problems have been reported with any of the other coded amino acid residues,‡ except for one report of *tert*-butyl ether cleavage and subsequent 2-oxazolidone, **39**, formation with MNP-Thr(Bu^t)-NH_2, **38**: scheme 12.[134]

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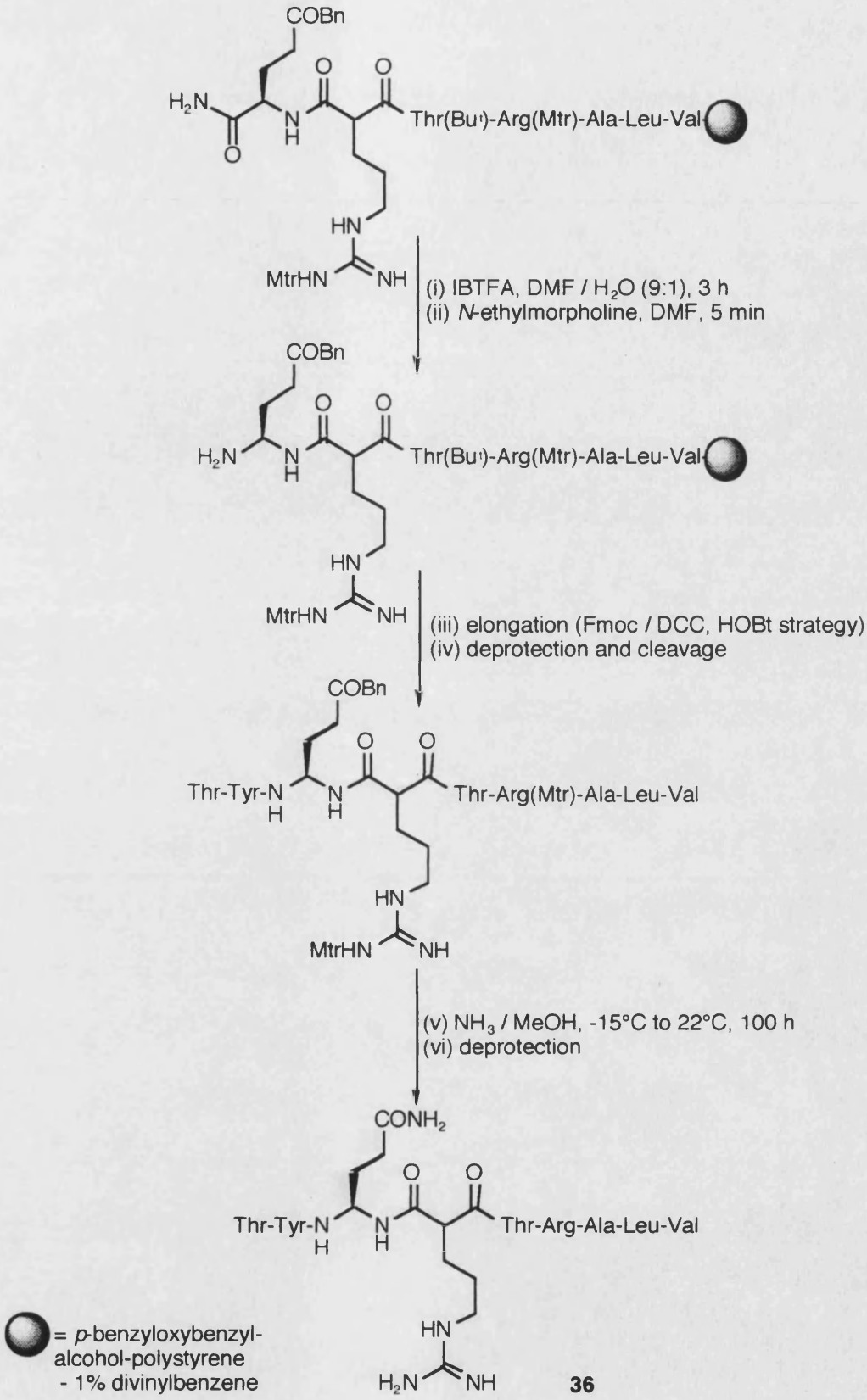
‡ However, IBTFA does oxidise free (unprotected) α-amino acids to the corresponding aldehyde (and nitrile in some cases), carbon dioxide and ammonium trifluoroacetate.[127]



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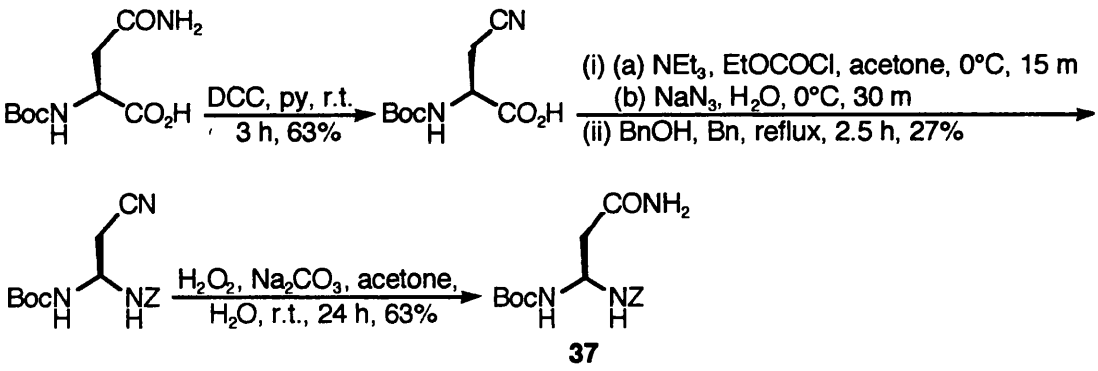
Scheme 10: Synthesis of the PMRI killer-cell epitope of influenza nucleoprotein, **36**.[133]



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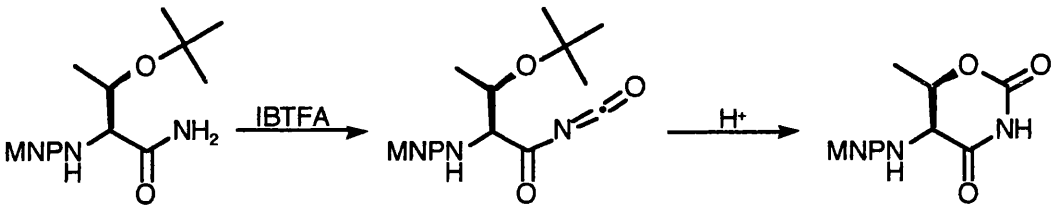
Scheme 11: Synthesis of Boc-*g*Asn-Z, **37**.[123]



38 **39**

Scheme 12: Acid catalysed deprotection of MNP-Thr(Bu^t)-NH_2, **38**, during IBTFA treatment.[134]

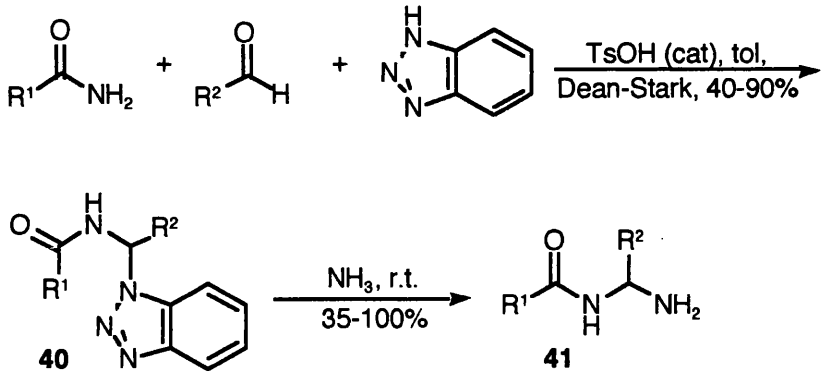
The IBTFA mediated Hofmann rearrangement is now the most frequently employed method of *gem*-diaminoalkyl derivative synthesis, with the above mentioned provisos. It is certainly the method of choice for the synthesis of peptidyl *gem*-diaminoalkyl derivatives.

(2) The Mannich reaction

Katritzky and co-workers synthesised racemic, mono-protected *gem*-diaminoalkyl compounds, **41**, via the Mannich reaction of an amide, aldehyde and benzotriazole (Bt) to generate the (isolated) intermediate adduct, **40**, and subsequent reaction with ammonia: scheme 13.[135] Use of *N*-protected α-amino acid amides as the amide component gave peptidyl *gem*-diaminoalkyl derivatives.[135] Although this procedure suffers from the drawback of unselectively producing both epimers (at the *gem*-diamino carbon) of the product, **41**,§ it has the advantage of expanding the range of *gem*-diaminoalkyl derivatives

§ Diastereoselectivity in the generation of the intermediate, **40**, was observed in some cases when an amino acid amide was employed. However, the Bt / NH_3 displacement step proceeded with complete racemisation, presumably via an S_N1 mechanism. The peptidyl *gem*-diamino alkyl compounds, **41**, were readily resolved during purification to provide diastereomerically pure products.[135]

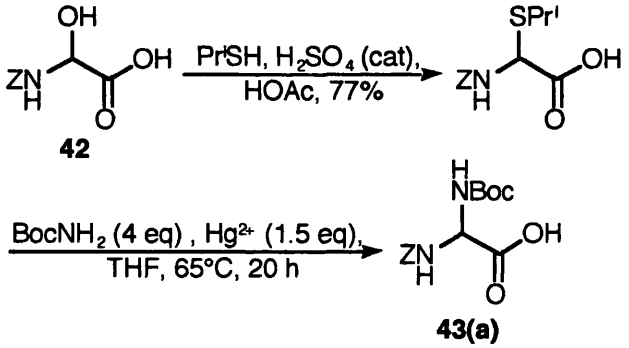
easily accessible beyond those derived from α-amino acids (*i.e.* variation of R^2 is more simple here than with a rearrangement method where synthesis of the appropriate α-amino acid is necessary to produce variation far beyond the 20 coded amino acid side chains).



Scheme 13: Mannich reaction leading to *gem*-diaminoalkyl compounds, **41**. In all cases small quantities of the benzotriazol-2-yl isomer of the intermediate, **40**, were also produced.[135]

(3) Displacement of sulfur

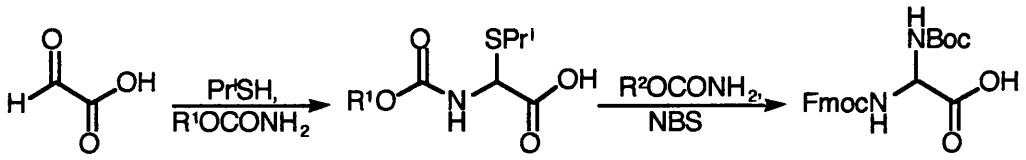
Bock and co-workers synthesised orthogonally protected α-aminoglycine, **43**, from α-hydroxy-Z-glycine, **42**.[136] Amidoalkylation of 2-propanethiol and subsequent mercuric ion mediated displacement of sulfur with *tert*-butyl carbamate yielded racemic α-Boc-amino-Z-glycine, **43(a)**: scheme 14.[136]



Scheme 14: Synthesis of orthogonally protected α-aminoglycine, **43**.[136] Standard protection, deprotection and coupling steps facilitated elaboration at either amino group.[136]

Similarly, Badet and co-workers synthesised α-Boc-amino-Fmoc-glycine, **43(b)**. A Mannich type reaction of a carbamate, 2-propanethiol and glyoxylic acid hydrate, **44**, furnished the intermediate **45**, which yielded racemic α-Boc-amino-Fmoc-glycine, **43(b)**, upon NBS mediated displacement of sulfur: scheme 15.[137] Badet and co-workers

employed α-Boc-amino-Fmoc-glycine, **43(b)**, to incorporate an α-amino-glycine residue into an octapeptide using SPS, although they did not use it to furnish a *gem*-diamino residue in a PMRI peptide.[137]

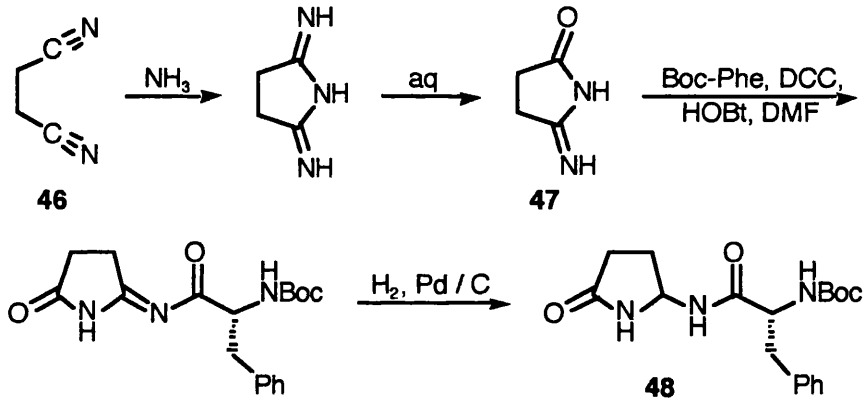


44 **45** **43(b)**

Scheme 15: Synthesis of orthogonally protected α-aminoglycine; R^1 = Bu^t, R^2 = 9-fluorenylmethyl, or *vice versa*.[137]

(4) From nitriles

Hayward and Morley's synthesis of the first *gem*-diaminoalkyl residue containing peptide, **48**, started from succinodinitrile, **46**.[98] They prepared 5-imino-pyrrolidin-2-one, **47**, by cold aqueous hydrolysis of the product from succinodinitrile, **46**, and ammonia; imino-acylation and subsequent hydrogenation yielded a (separable) mixture of the desired pseudodipeptide diastereomers, **48**: scheme 16.[98]

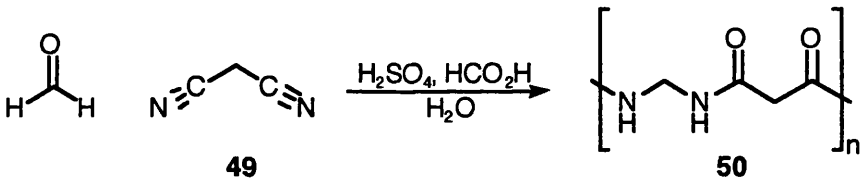


46 **47** **48**

Scheme 16: Synthesis of Boc-Phe-(*R,S*)*g*Glp, **48**.[98]

Though theoretically general, no method based on the reduction of acylamidines, such as **47**, has since found use in PMRI peptide synthesis.

Finally, although not generally applicable to the synthesis of PMRI peptides, Puiggalí and Muñoz-Guerra's synthesis of nylon 1,3 {*i.e.* $[Gly\psi(NHCO)Gly]_n$}, **50**, from malononitrile, **49**, and formaldehyde using Magat's formic acid / sulfuric acid catalysed polymerisation,[138] is worthy of note: scheme 17.[139]

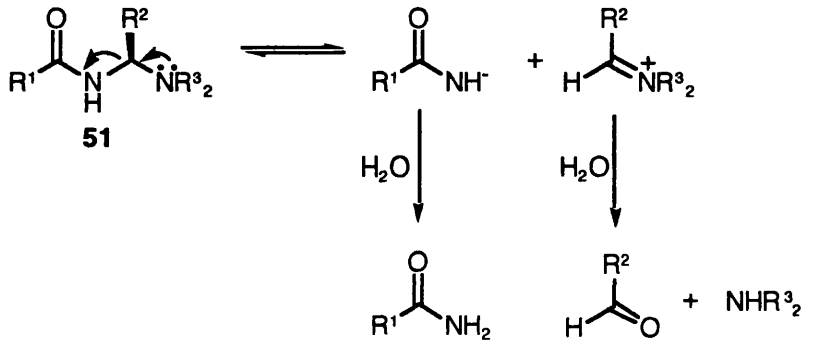


Scheme 17: Synthesis of nylon 1,3, **50**.

The literature contains other methods of *gem*-diamino derivative synthesis, but the resultant derivatives are unsuitable for incorporation into PMRI peptides.

(5) Decomposition

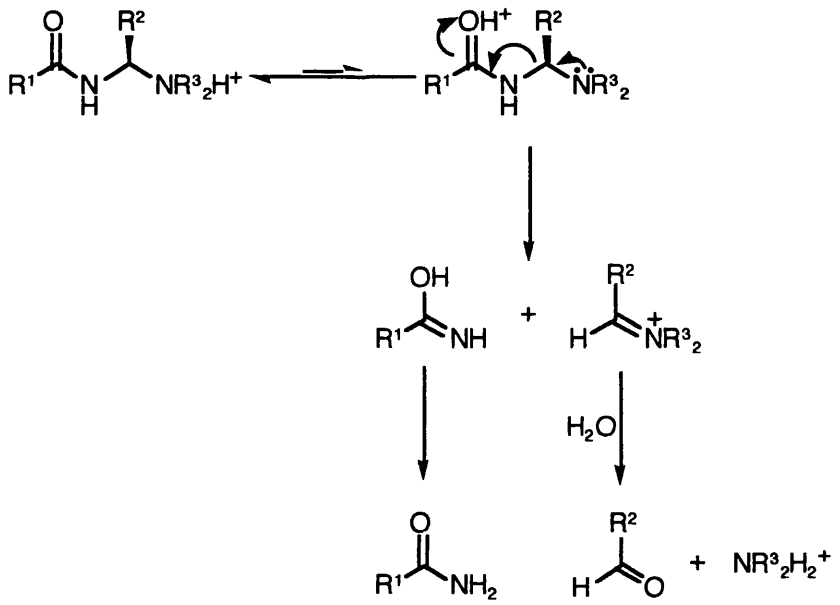
Loudon and co-workers, following on from their work on carboxy-terminal peptide degradation mentioned above, studied the mechanism of hydrolysis of mono-protected *gem*-diaminoalkyl compounds, **51**.[116,¶] Under basic conditions the mechanism of hydrolysis is as shown in scheme 18, and the observed rates of hydrolysis were in the order **51(d)** > **51(b)** > **51(a)**, due to imine stabilisation in **51(d)** and a better leaving group in **51(b)** than in **51(a)**.[116]



Scheme 18: Hydrolysis of *gem*-diaminoalkyl compounds under basic conditions; **(a)** R^1 = Me, R^2 = Pr^i, R^3 = H; **(b)** R^1 = $MeOCH_2$, R^2 = Pr^i, R^3 = H; **(c)** R^1 = Bu^tO, R^2 = Pr^i, R^3 = H; and **(d)** R^1 = Me, R^2 = Pr^i, R^3 = Me.[116]

Under acidic conditions the mechanism shown in scheme 19 operates, with a possible transition state illustrated in figure 10.[116]

¶ This is the only study of the decomposition of *gem*-diamino alkyl compounds of the variety encountered in PMRI peptides. Other researchers have studied the decomposition of *gem*-diamino alkyl moieties in different chemical contexts.[140,141]



Scheme 19: Hydrolysis of *gem*-diaminoalkyl compounds under acidic conditions; R^1, R^2 and R^3 as scheme 18.[116]

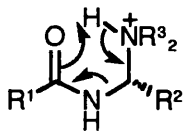


Figure 10: Possible transition state.[116]

The study of *gem*-diaminoalkyl compound **51(c)** was limited due to its instability: under acidic conditions it was hydrolysed 9.2 times faster than **51(a)** (at 50°C).[116] This rate acceleration is due to the better leaving group in **51(c)** overcoming its lower basicity.[116]

These mechanisms suggest that *gem*-diaminoalkyl compounds **51** with R^2 = H should be more stable.[116] The results enable an estimate of 10-50 h for the biological half-life of **51(a)**,[116] and reiterate the advantage of synthesising peptidyl rather than mono-(carbamate) protected *gem*-diaminoalkyl derivatives.[116]

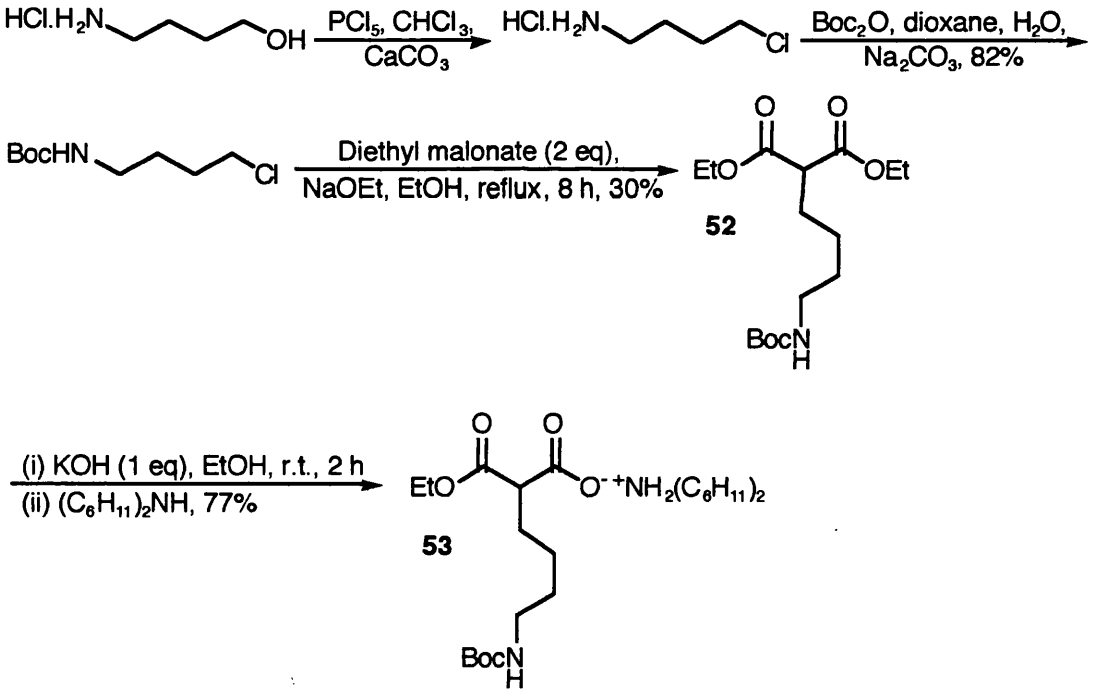
Overall the stability of monoacyl *gem*-diaminoalkyl compounds is much the same at mildly acidic and mildly basic pH, and is greater than one might expect.[116] These mechanisms explain this stability: under basic conditions the leaving group (an amide anion) is poor, whereas under acidic conditions an unfavourable equilibrium must be overcome.[116]

(ii) C^2 Substituted malonyl derivatives

Considerations for the coupling and direct incorporation of malonyl derivatives were discussed above [section **1.5.1***(a)(i)(1)*]. Here we consider the preparation of C^2 substituted malonyl derivatives appropriate for incorporation into PMRI peptides. The derivatives required are substituted at C^2 with an amino acid side chain and are mono-protected, *e.g.* **53**.

(1) Alkylation and partial hydrolysis of malonic acid diesters

This classical method provides access to many appropriate C^2 substituted malonyl derivatives, *e.g.* those corresponding to Phe, Lys, Trp, Leu, Ala, His and Met.[108,121] Deprotonation of malonic acid diesters with sodium ethoxide inhibits dialkylation by most electrophiles (because the monoalkyl derivative, *e.g.* **52**, is less acidic than ethanol).[142] Partial saponification of the resultant C^2 substituted diesters yields the corresponding monoesters: *e.g.* scheme 20.

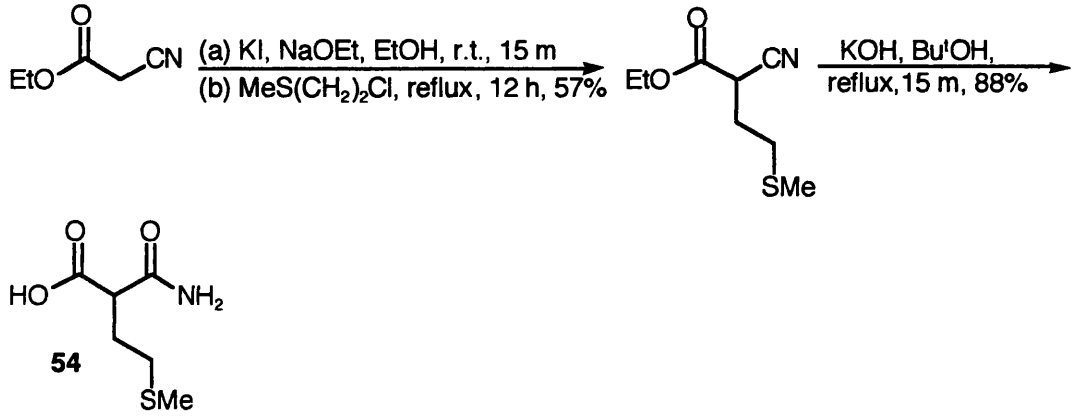


Scheme 20: Synthesis of (*R,S*)*m*Lys(Boc)-OEt, **53**, for incorporation into PMRI somatostatin, by alkylation of diethyl malonate. Overall yield 19%.[143]

The recent development of the allyl group as a protecting group for the C^2 hydrogen of malonates may facilitate the extension of this method to classically more challenging side chains.[144]

(2) Alkylation and hydrolysis of cyanoacetates

C^2 substituted malonamic acids, suitable only for carboxy terminal incorporation, may be synthesised by alkylation and hydrolysis of cyanoacetates, *e.g.* scheme 21, however dialkylation is a more serious problem here, due to the substrates' increased acidity.[142]

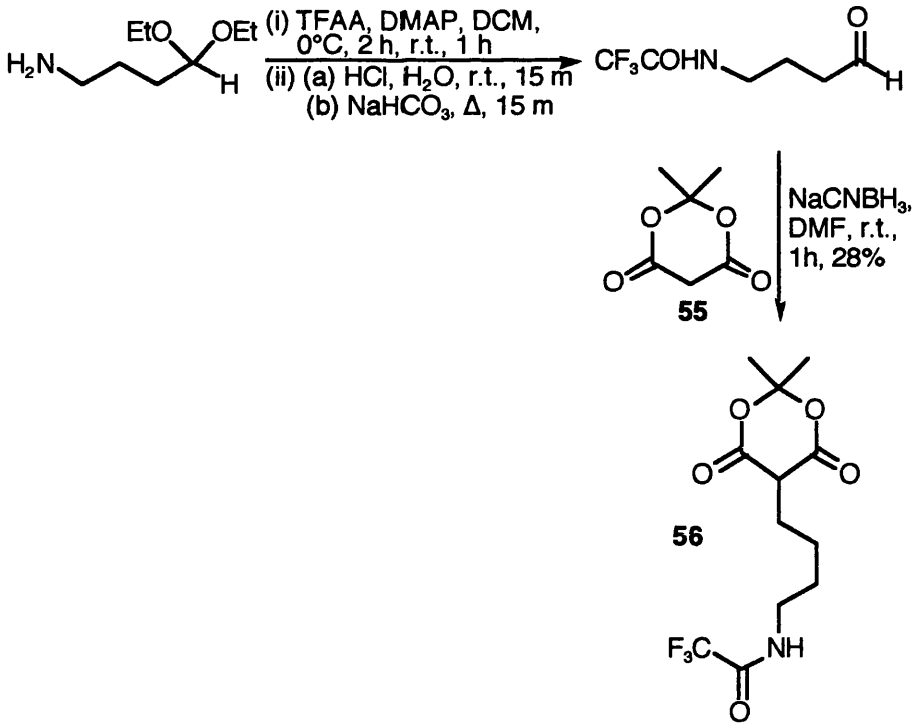


Scheme 21: Synthesis of *m*Met-NH_2, **54**, for incorporation into end group modified bombesin *C*-terminal nonapeptide. The product, **54**, existed in equilibrium with both enol tautomers, but this did not prevent subsequent peptide coupling using standard methods.[123]

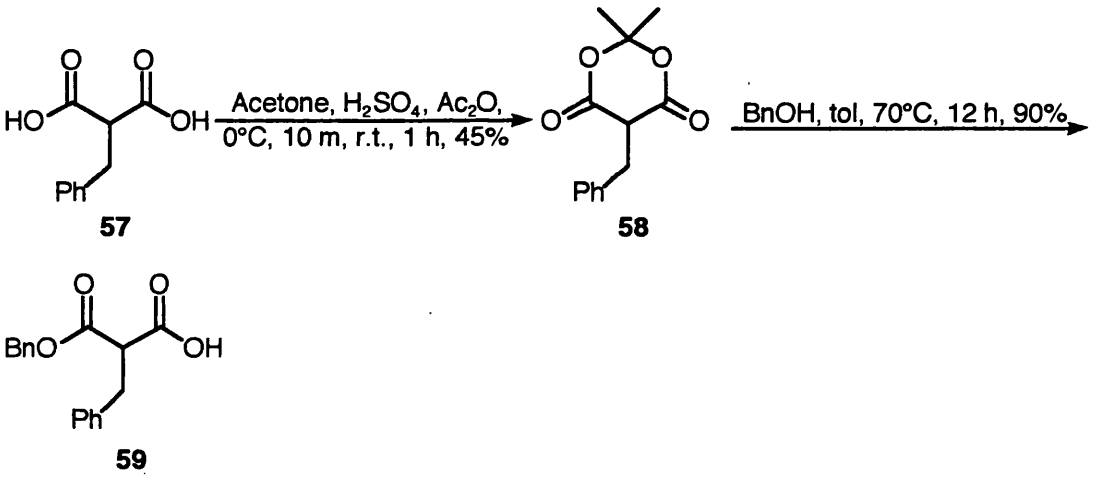
(3) From Meldrum's acid

Suitable side chains may be introduced at C^5 of Meldrum's acid, **55**, by (1) reductive alkylation, or (2) a two step process via an alkylidene or a cyclopropyl derivative.[126,145]

Knoevenagel reaction of Meldrum's acid, **55**, with aldehydes (or some ketones) and reduction by a boron reductant (*in situ* with borane.dimethylamine complex, borane.trimethylamine complex, or sodium cyanoborohydride; subsequently with sodium borohydride) yields mono C^5 substituted Meldrum's acids, *e.g.* scheme 22.[146-148] Subsequent alcoholysis yields C^2 substituted malonic acid monoesters. Indeed, this alcoholysis is sufficiently convenient that Chorev, Goodman and co-workers prepared the Meldrum's acid derivative of 2-benzylmalonic acid, **57**, in order to obtain the corresponding malonic monoester, **59**, by ring opening with benzyl alcohol: scheme 23.[149]



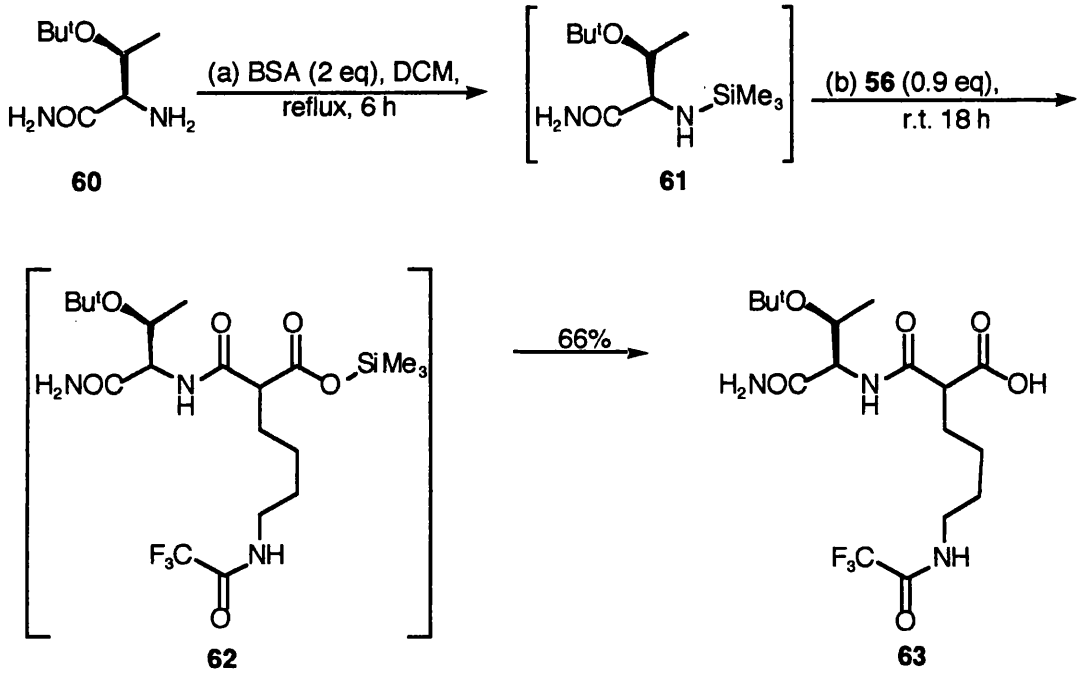
Scheme 22: Synthesis of Meldrum's acid derivative **56**, corresponding to *m*Lys(TFA), for incorporation into PMRI tuftsin.[146]



Scheme 23: Synthesis of monobenzyl 2-benzyl malonate, **59**, by alcoholysis of the corresponding Meldrum's acid derivative, **58**.[149]

Alternatively, mono C^5 substituted Meldrum's acids may be ring opened with silylated amines.[150] Silylation prevents the amine acting as a base and deprotonating the Meldrum's acid derivative at C^5.[146] Thus the Meldrum's acid derivative remains in the keto form appropriate for the ring opening reaction. The reaction mechanism is complex and probably involves a cyclic transition state which utilises a vacant silicon 3d-orbital.[150,151] Verdini and co-workers used this approach to ring open Meldrum's acid derivatives with amino acids or mono-protected *gem*-diaminoalkyl compounds which they

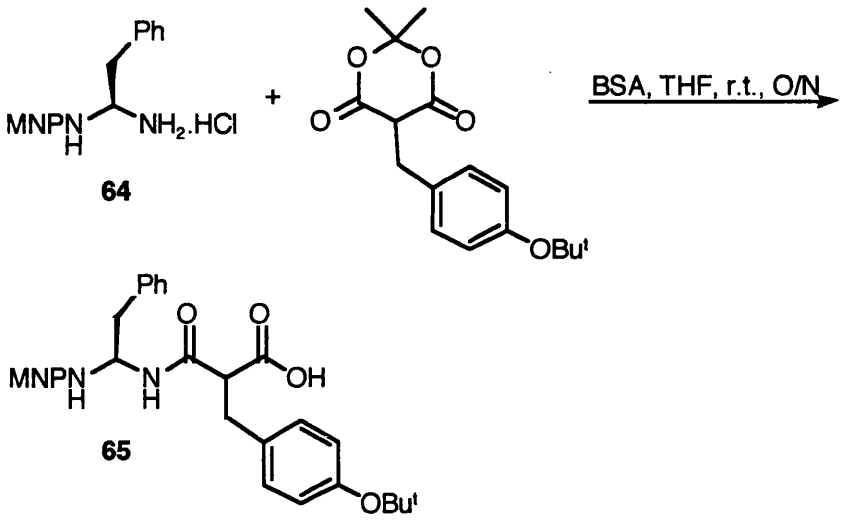
silylated, in the same pot, with *O,N*-bis(trimethylsilyl)acetamide (BSA).[126,146,152,153] Thus they prepared H_2N-D-*r*Thr(Bu^t)-*m*Lys(TFA), **63**, scheme 24, which they subsequently incorporated into PMRI tuftsin.[146]



Scheme 24: Synthesis of H_2N-D-*r*Thr(Bu^t)-*m*Lys(TFA), **63**. This was a one-pot reaction and the depicted intermediates, **61** and **62**, were not isolated or characterised.[146] It is distinctly possible that D-Thr(Bu^t)-NH_2, **60**, was bis-silylated under the reaction conditions (*i.e.* as **61** but with a second TMS group on the amide nitrogen).[150,152] The silylated intermediate **62** was hydrolysed during work-up. Note that C^β of -D-Thr- was not inverted during this procedure.

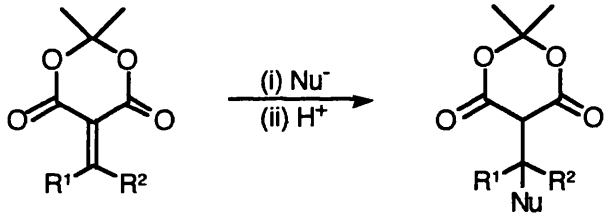
Verdini and co-workers also used this method to prepare the PMRI dipeptide MNP-Pheψ(NHCO)(*R,S*)Tyr(Bu^t), **65**, scheme 25, having demonstrated that trimethylsilylation does not adversely affect the stability of MNP-*g*Phe.HCl, **64**.[126]

Dal Pozzo and co-workers, in their synthesis of a PMRI analogue, **67**, of the growth factor Gly-His-Lys, compared BSA mediated amidative ring opening with alcoholysis and subsequent coupling of the Meldrum's acid C^5 derivatives **66(a)** and **66(b)**, respectively: scheme 26 (p. 56).[155] Both routes gave similar yields (31% and 24% respectively).[155]



Scheme 25: Synthesis of MNP-Pheψ(NHCO)(*R*,*S*)Tyr(Bu^t), **65**, for use in SPS.[126] Excess BSA was used, and no base was necessary.[126,152,154] Again, the silylated species was not isolated or characterised.

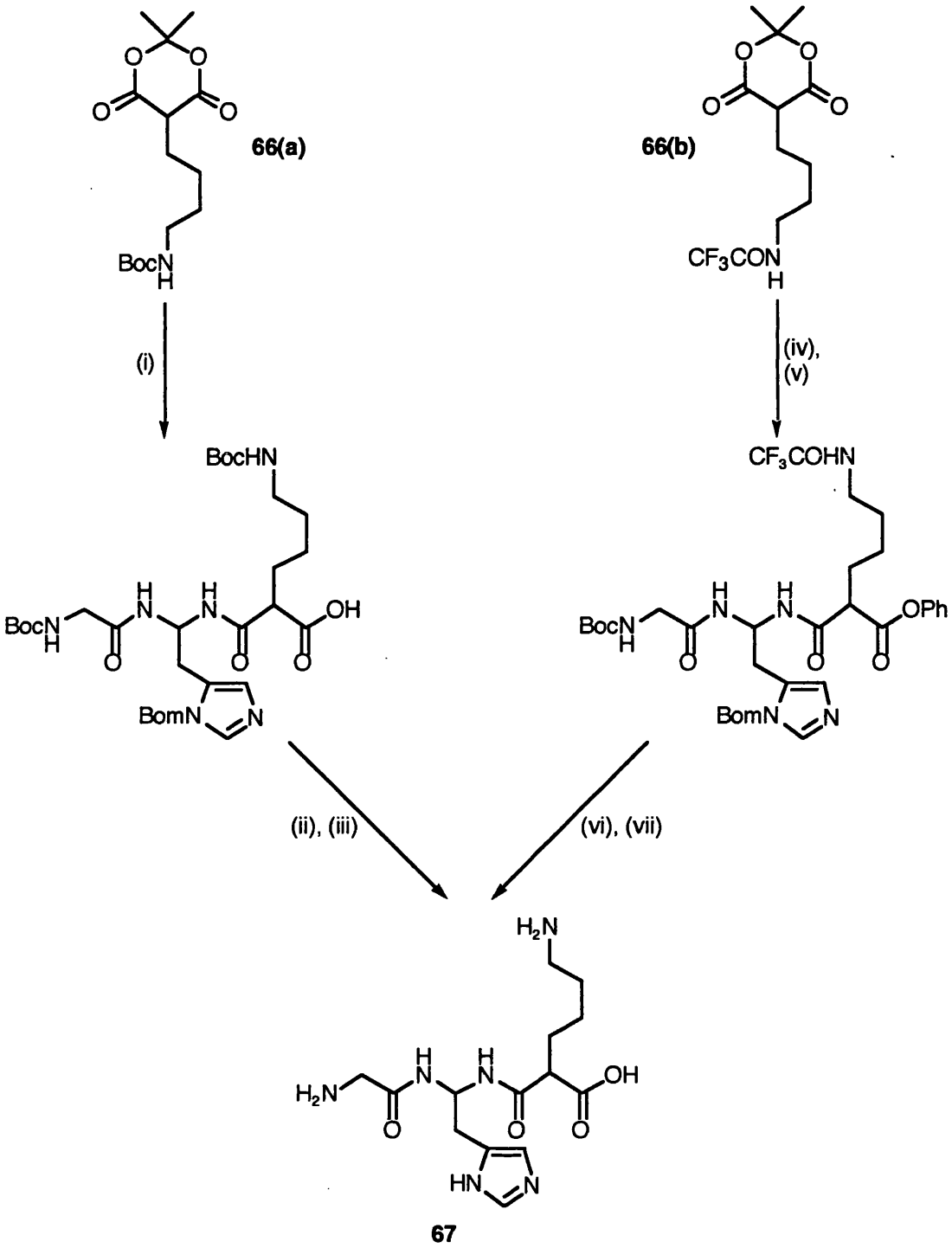
Nucleophilic addition to Meldrum's acid Knoevenagel products, **68**, permits access to C^5 derivatives, **69**, suitable for elaboration as above: scheme 27.[126,145]



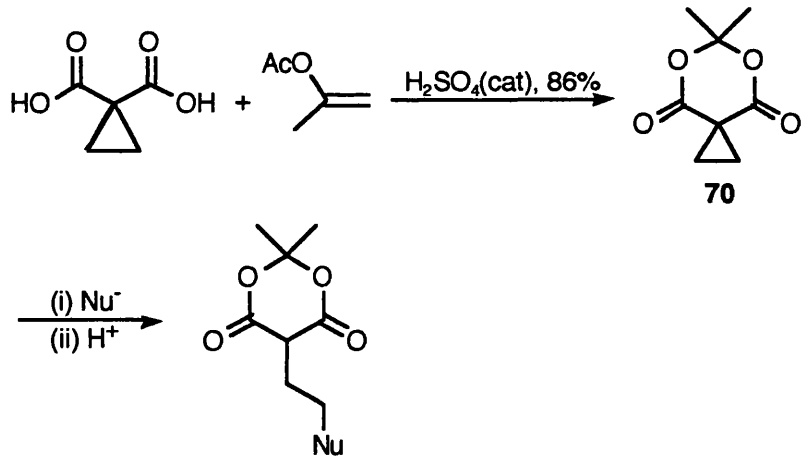
68 **69**

Scheme 27: Synthesis of *m*Xaa precursors.

Alternatively C^5 cyclopropyl derivatives, **70**, may be prepared and similarly attacked with nucleophiles: scheme 28.[145,156]



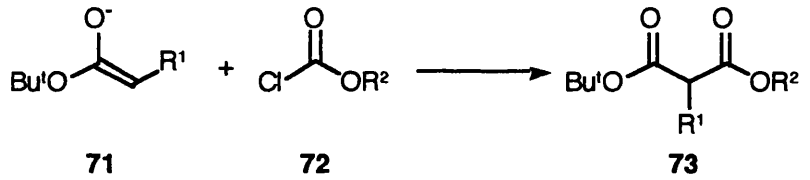
Scheme 26: Alternative routes to Gly-Hisψ(NHCO)(*R*,*S*)Lys, **67**. (i) Boc-Gly-*g*His(Bom), BSA, DCM, reflux, 6 h, then **66(a)**, r.t., O/N. (ii) TFA, r.t., 20 m, 79%. (iii) Thioanisole, TFA, TMSOTf, 0°C, 30 m, 53%. (iv) PhOH, 110°C, 2.5 h + 15 m under reduced pressure, 74%. (v) Boc-Gly-*g*His(Bom), DCC, HOBt, py, DCM, 0°C, 5 m, r.t., 3 h, 63%. (vi) NaOH, H_2O, MeOH. (vii) NaI, TMSCl, MeCN, 80°C, 6 h, 51%.[155]



Scheme 28: Synthesis of *m*Xaa precursors via Meldrum's acid C^5 cyclopropyl derivative, **70**.[145,156]

(4) Acylation of tert-*butyl carboxylates*

Acylation of the enolate of a *tert*-butyl carboxylate, **71**, with an alkyl or benzyl chloroformate, **72**, yields an orthogonally protected, C^2 substituted malonate, **73**: scheme 29.[69]

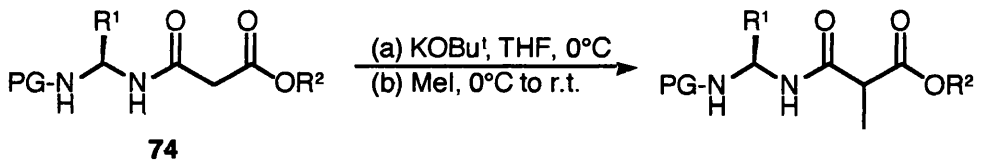


71 **72** **73**

Scheme 29: Synthesis of Bu^tO-*m*Xaa-OR^2, **73**. R^1 = suitable amino acid side chain, R^2 = alkyl or benzyl.

(5) Alkylation of PMRI dipeptides

Campbell and co-workers found that the yield of Goldschmidt and Wick type reactions using C^2 substituted monomalonates was lower than when using C^2 unsubstituted monomalonates.[84] Thus they introduced the desired side chains (*i.e.* the malonate C^2 substituents) by specific alkylation of the PMRI dipeptides PG-Xaaψ(NHCO)Gly-OR^2, **74**: *e.g.* scheme 30.[84]

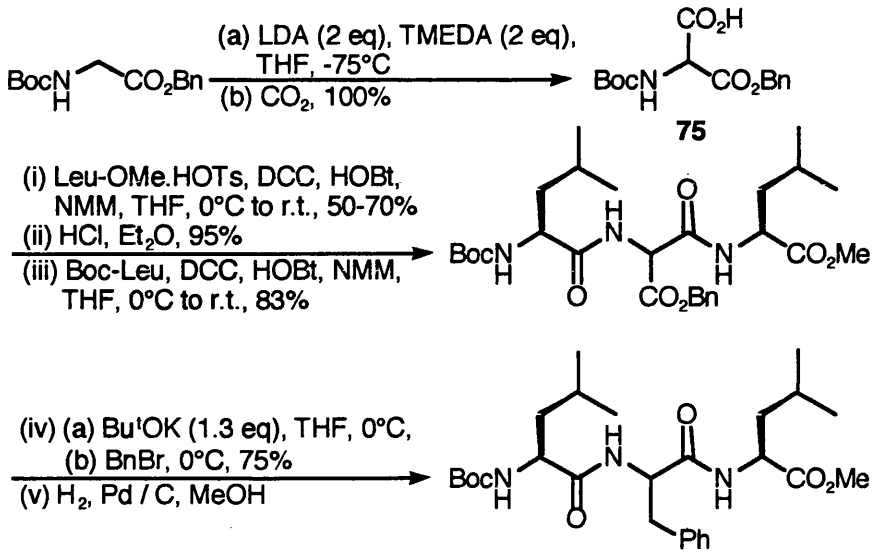


74

Scheme 30: Alkylation of PMRI dipeptides containing -*m*Gly- to produce -*m*Ala-; PG = Ac, Boc or Z; R^1 = amino acid side chain; and R^2 = Et or Ph.[84,157] Note that this is a special case of the alkylation of malonic acid esters, discussed in section **1.5.1***(a)(ii)(1)*.

(6) Carboxylation of glycine

The simplicity of malonate alkylation [*(1)* and *(5)* above] is demonstrated by Seebach's recent introduction of a malonyl moiety into a peptide in order to facilitate specific C-alkylation: *e.g.* scheme 31.[158]



Scheme 31: Peptide C-alkylation facilitated by an aminomalonate moiety.[158]

This method necessitated the synthesis of C^2 malonyl derivative **75**, which does not, however, correspond to a coded amino acid when viewed from the PMRI peptide perspective. This approach is conceptually very similar to that described in section **1.5.1***(a)(ii)(4)* above.

(7) Configurational lability: C^2 fluorinated and bis-alkylated malonyl derivatives

None of the above syntheses of C^2 substituted malonyl derivatives are stereoselective: all yield both epimers at C^2. However, this lack of selectivity is not a serious consideration because the C^2 malonyl position is configurationally labile during synthesis.*,[69] Many PMRI peptide epimers (at the C^2 malonyl position) have been separated by RPHPLC and the half-lives for the epimerisation measured.[69] The half-lives vary from minutes to days, with cyclic PMRI peptides generally displaying greater stability.[69] Since the epimerisation occurs (with general acid-base catalysis) via a

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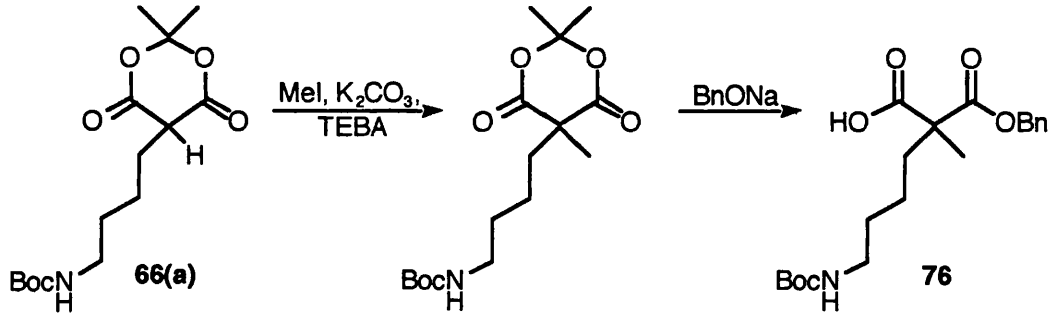
* C^2 substituted malonamic acids are more configurationally stable than C^2 substituted malonic acids,[123] therefore the configurational stability of malonyl residues increases upon their incorporation into PMRI peptides.

coplanar enol structure, the extra stability of cyclic PMRI peptides can be understood in terms of steric hindrance (which blocks the deprotonation) and / or conformational strain (which disfavours the coplanar arrangement).[69,159] Because the epimers of PMRI analogues of peptides often display significantly different biological activities, and, indeed, only one can exhibit full topochemical complimentarity with the parent peptide, the assignment of configuration is important. Several methods of configurational assignment (which utilise RPHPLC retention times, degradation, ^{1}H NMR chemical shifts, or NOEs) have been developed.[69] However, it must be borne in mind that, particularly in the case of epimers with short half-lives, separation and assignment may be futile, for subsequent epimerisation (the extent of which is difficult to predict) under assay conditions is a possibility, leading to blurred bioactivity results.[159]

Further derivatisation of the C^2 malonyl position overcomes the configurational lability.

Dal Pozzo and Laurita prepared a racemic 2-methyl-2-alkyl malonyl residue: scheme 32.

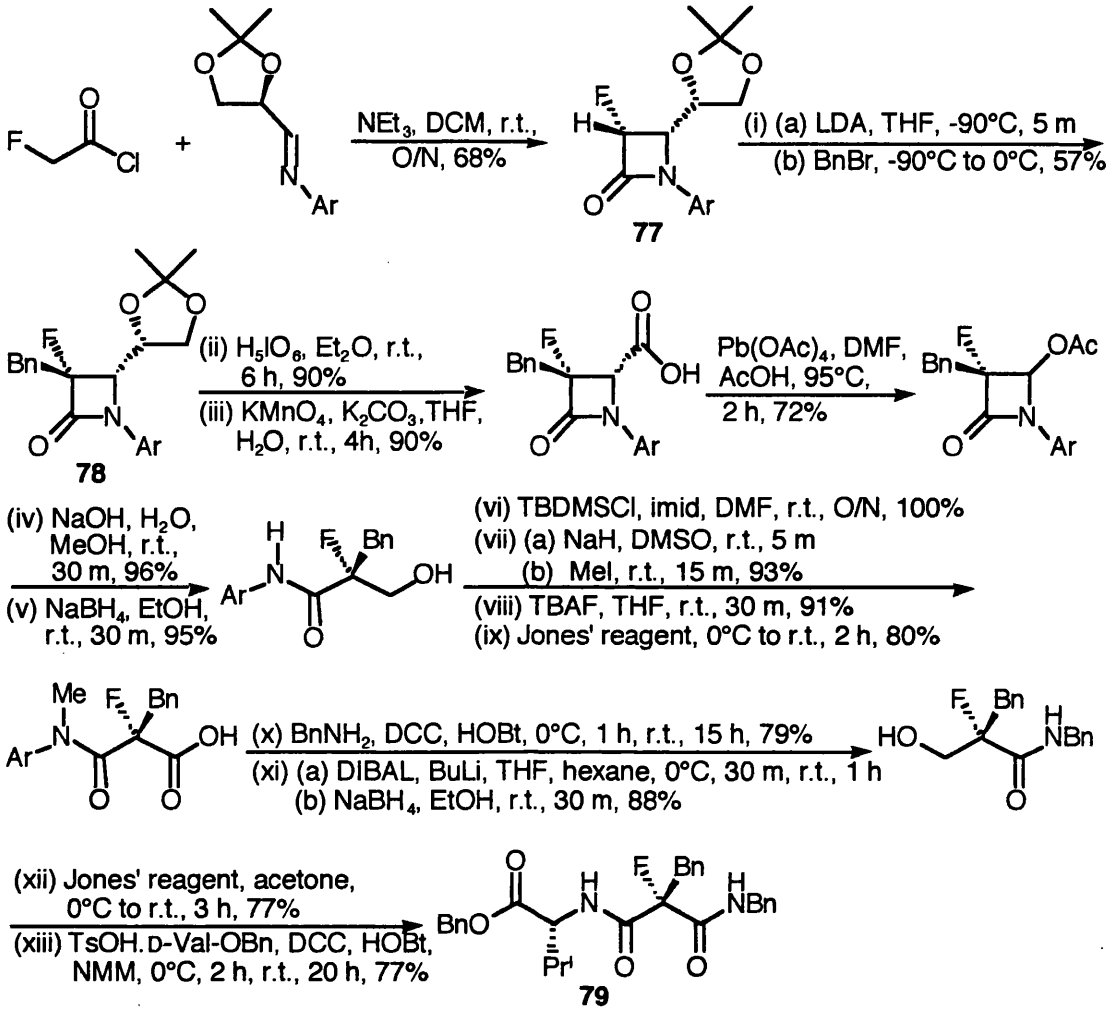


Scheme 32: Preparation of 2-Me-*m*Lys(Boc)-OBn, **76**.[160]

They incorporated the racemate into a PMRI growth factor, analogous to **67**, and separated the diastereomers by HPLC.[160] The resultant PMRI growth factor retained biological activity.[160]

Welch and co-workers replaced the labile malonyl C^2 proton with fluorine, resulting in an optically pure and configurationally stable 2-fluoro-2-benzyl malonyl residue which was incorporated into a (proposed) HIV-1 protease inhibitor, **79**.[161] The synthesis was somewhat tortuous: scheme 33; this idea has yet to be adopted by other

researchers, but may prove to be very useful since a wide range of 3-fluoro β-lactams, analogous to **78**, are available by stereoselective alkylation or aldol reaction of **77**.[162]



Scheme 33: Synthesis of a PMRI peptide containing a configurationally stable fluoro-malonyl residue (-*mF*Phe-). Ar = *p*-methoxy-phenyl.[161,162]

(b) Solid phase methodology

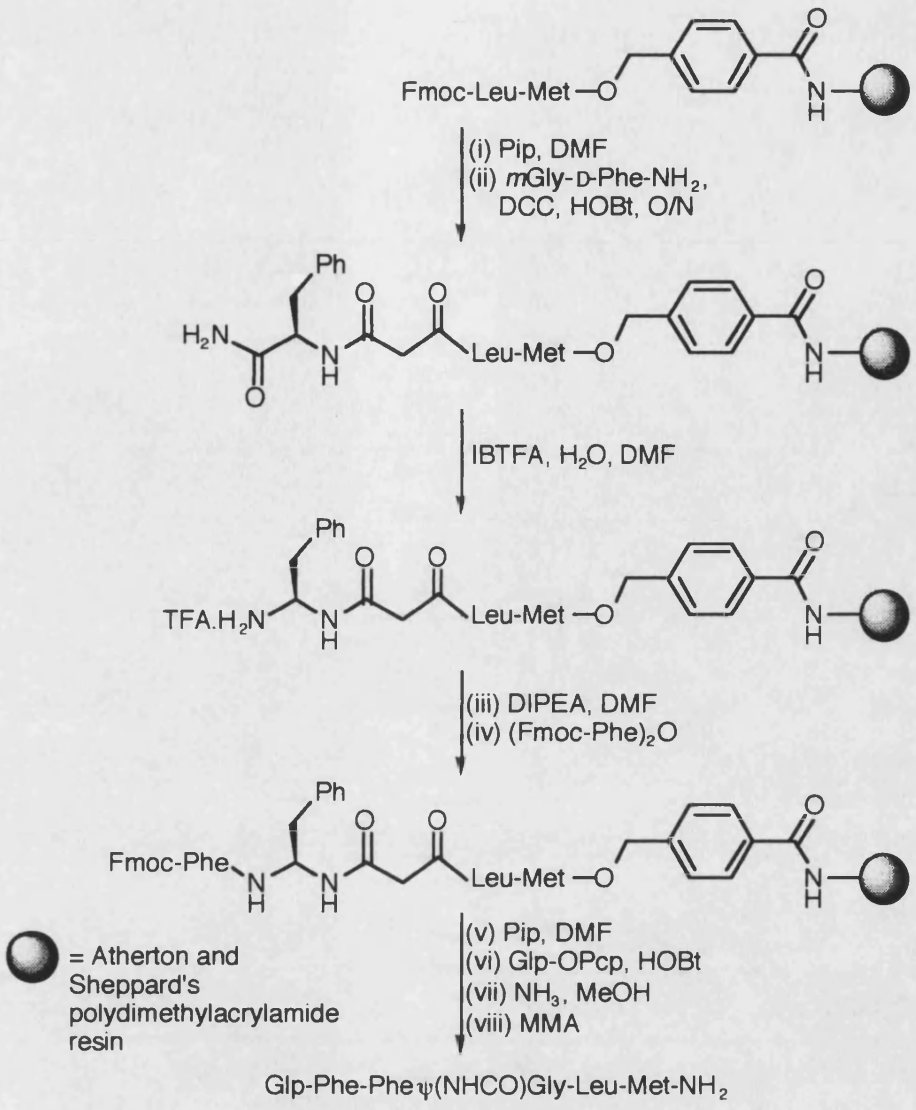
SPS of peptides employs a solid, polymeric resin as a special variety of carboxy terminal protecting group, to which the growing peptide chain remains attached until the end of the synthesis. Each residue is introduced (in amino terminal protected form) using some manner of carboxy terminus activation. Following amino terminal deprotection of the resin bound (pseudo)peptide, the cycle is repeated. Thus special considerations for SPS of PMRI peptides essentially concern the compatibility of the usual solution phase methodology with the resin.

In this section significant examples of PMRI peptide SPS are considered, which demonstrate the compatibility (or otherwise) of the solution phase methodology with the resin bound conditions, and which introduce new or modified methodology into the SPS arena.

(i) IBTFA

Verdini and co-workers performed the first PMRI peptide SPS,[163] and have been the major contributors to the development of PMRI peptide SPS. Their pioneering synthesis of the PMRI substance P analogue, **80**, demonstrated the compatibility of IBTFA with the solid support (which is also displayed in scheme 10): scheme 34. It is noteworthy that the incorporation of malonyl-D-phenylalanine amide [step (ii), scheme 34] required an unusually long reaction time and resulted in a *ca.* 20% loss of peptide, probably due to Leu-Met diketopiperazine formation.[163] This is a symptom of the reduced coupling efficiency of malonates, mentioned above [section **1.5.1***(a)(i)(1)*]. The final treatment with MMA was necessary to reverse the effect of IBTFA on -Met-, *i.e.* to reduce methionine sulfoxide back to -Met-.[163]

Verdini and co-workers used a similar procedure to synthesise the PMRI bradykinin potentiating peptide analogue, Glp-Trp-Pro-Arg-Pro-Lysψ(NHCO)(*R*,*S*)Phe-Ala-Pro (in 25% yield), which exhibited *in vivo* activity and enhanced resistance towards cleavage by ACE *in vitro*.[164] However, in order to avoid substantial Ala-Pro diketopiperizine formation during malonyl coupling, the malonyl residue was incorporated as Ala-(*R*,*S*)*m*Phe-D-Lys(Boc)-NH_2.[164]



80

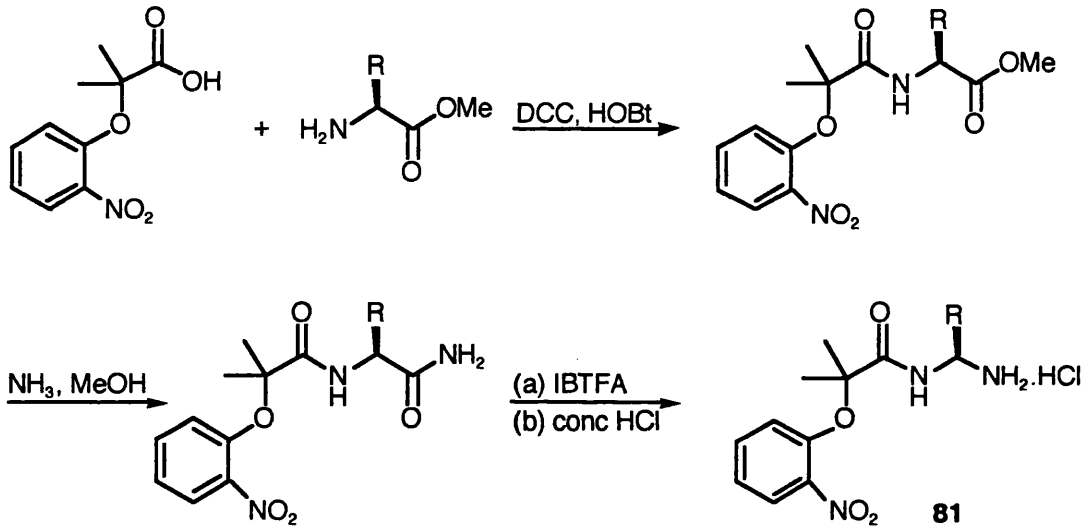
Scheme 34: SPS of a PMRI substance P analogue, **80**.[163] Note the direct synthesis of the PMRI dipeptide unit using IBTFA (as in scheme 9).

(ii) The MNP group

Although the treatment of a resin bound malonyl-D-amino acid amide with IBTFA, described above [section **1.5.1***(b)(i)*], is a useful method, it suffers from the previously discussed residue specific problems inherent to IBTFA [section **1.5.1***(a)(i)(1)*], plus the rather high expense of D-amino acids. It is therefore desirable (and may be essential) to avoid treatment of a growing, resin bound peptide chain containing Asn, Gln, Met, Cys, Tyr or Trp, with IBTFA if side-reactions and / or extra synthetic manipulations are to be avoided. This objective is readily achieved by incorporation of preformed PMRI dipeptides, PG-Xaaψ(NHCO)Yaa (or the appropriate *gem*-diaminoalkyl residue

containing fragment for the synthesis of PMRI peptides with a consecutive sequence of reversed bonds).† Therefore, suitably protected PMRI dipeptides are required. Difficulties in synthesising carbamate protected PMRI dipeptides are discussed above [section **1.5.1***(a)(i)(1)*]. Boc protected PMRI dipeptides are accessible[84] and compatible with Merrifield SPS, but have not been utilised. Verdini and co-workers found that mono Fmoc, trifluoroacetyl or diphenylphosphinoyl-*gem*-diamino alkanes were insufficiently stable to be useful for PMRI dipeptide synthesis.[126] They therefore searched for a new protecting group and discovered that the (2-methyl-2-*o*-nitrophenoxy)-propionyl (MNP) group is appropriate.[126]

MNP protected *gem*-diamino alkanes may be synthesised as shown in scheme 35.

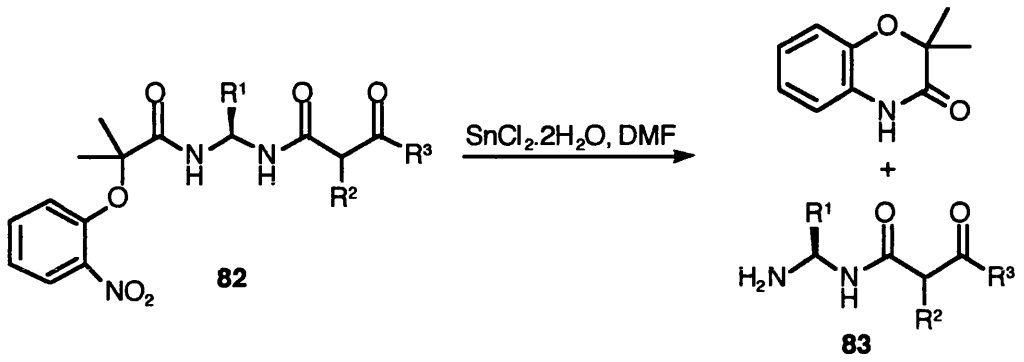


Scheme 35: Synthesis of MNP-*g*Xaa.HCl, **81**.[126]

The resultant mono MNP-*gem*-diamino alkanes are stable for prolonged periods as their hydrochlorides, **81**.[126] When deprotonated they are sufficiently stable to be acylated (DCC / HOBt) by mono esters of (C^2 substituted) malonic acids, and saponified to furnish the desired PMRI dipeptides.[126] Alternatively, *in situ* trimethylsilylation with BSA facilitates acylation by Meldrum's acids, as described above [section **1.5.1***(a)(ii)(3)*: scheme 25].

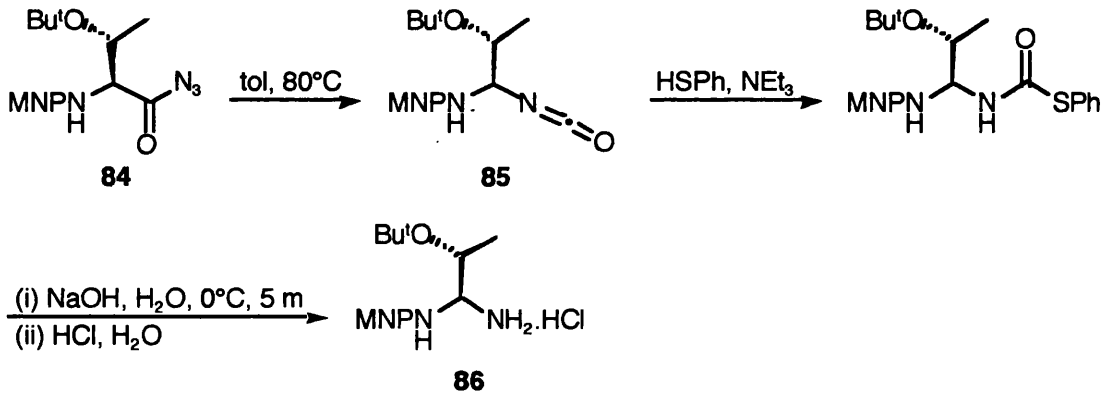
† None of the other methods of *gem*-diamino alkyl compound synthesis, successful in solution, have been tried in SPS, presumably due to excessive synthetic complications.

After incorporation of the MNP-PMRI dipeptide into the resin bound peptide, the MNP group is removed by treatment with tin(II) chloride in DMF, conditions compatible with Atherton and Sheppard's SPS protocol (using either acid or base labile linkers), leaving the resin bound PMRI peptide, **83**, ready for further elaboration: scheme 36.



Scheme 36: Deprotection of MNP-Xaaψ(NHCO)Yaa-R^3, **82**.[126] R^3 = resin bound peptide chain.

As shown in scheme 12, Verdini and co-workers encountered a problem when they tried this approach to MNP-*g*Thr(Bu^t).HCl, **86**. However, the desired compound was obtained from the acyl azide, **84**, using thiophenol to trap the intermediate isocyanate, **85**: scheme 37.[134]



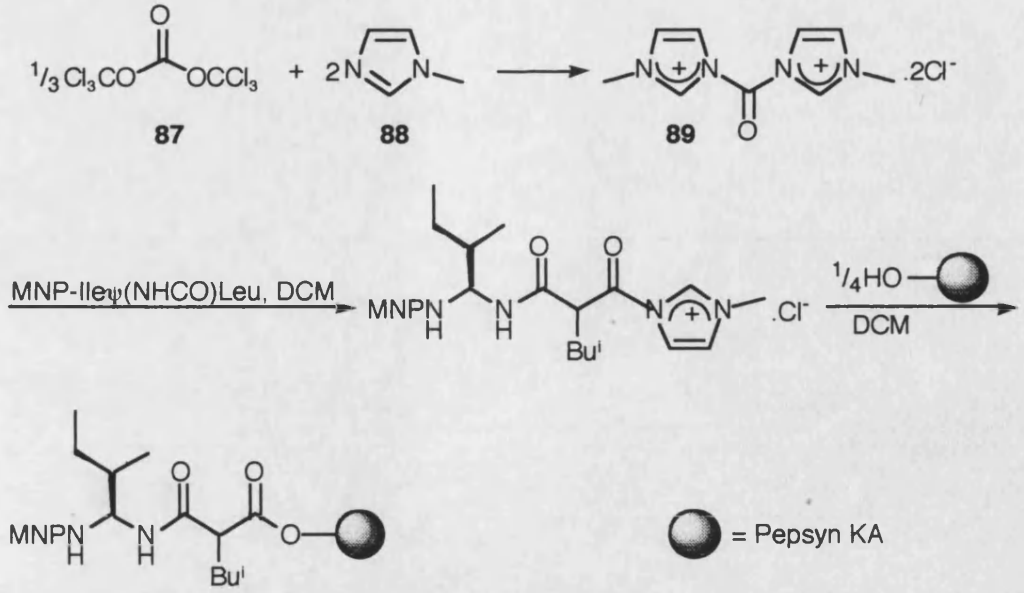
Scheme 37: Synthesis of MNP-*g*Thr(Bu^t).HCl, **86**, avoiding 2-oxazolidone, **39**, formation (*c.f.* scheme 12).[134]

This procedure has another advantage over the IBTFA method in that the resultant -*g*Thr- derivative, **86**, is topochemically equivalent to -Thr- [when -*g*Thr- is obtained from -D-Thr-NH_2 by the action of IBTFA (*c.f.* scheme 24) then its C^β stereochemistry is non-equivalent]. The same topochemical considerations favour this procedure for -*g*Ile-.[69]

MNP-*g*Ile- caused a different problem during MNP removal. Verdini and co-workers experienced incomplete removal of MNP under their standard deprotection conditions, and resultant by-product formation during the SPS of the PMRI NT(8-13) analogue, Arg-Arg-Pro-Tyr-Ileψ(NHCO)(*R*,*S*)Leu.[165] They ascribed this difficulty to steric hindrance from the Ile side chain and employed a longer deprotection time to minimise (though not eradicate) the problem.[165]

(iii) Anchorage

When preparing a PMRI peptide with a carboxy terminal malonyl residue by SPS, it is necessary to attach ("anchor") the malonyl residue to the solid support (via a linker). Therefore an ester linkage (if the target pseudopeptide has a free carboxy terminus) between the malonyl residue and the resin must be made. Under these conditions the reduced coupling efficiency of malonates [see section **1.5.1***(a)(i)(1)*] poses a significant challenge: Verdini and co-workers obtained yields of less than 15% when anchoring MNP-Ileψ(NHCO)(*R*,*S*)Leu to the Kieselguhr supported hydroxymethyl polyamide resin Pepsyn KA (using DCC / HOBt or BOP, with or without DMAP).[165] Furthermore, model esterifications of MNP-Ileψ(NHCO)(*R*,*S*)Leu with *p*-methoxybenzyl alcohol also gave poor yields.[165] Their solution to this problem was the *in situ* generation of the active coupling reagent carbonyl bis(*N*-methylimidazolium) dichloride, **89**, from triphosgene, **87**, and *N*-methylimidazole, **88**, which gave anchorage yields of 50-70%: scheme 38.[165] Subsequent elongation (Fmoc strategy) and cleavage - deprotection yielded the desired, agonistic, PMRI NT analogues.[165]



Scheme 38: Anchoring MNP-Ileψ(NHCO)(*R,S*)Leu using bis(*N*-methylimidazolium) dichloride, **89**.[165]

1.5.2 *Biologically active PMRI peptides.*

Many biologically relevant PMRI (and end group modified retro-inverso) peptides have been synthesised and tested; most are hormone analogues, but there are some examples of protease inhibitors and sweeteners. Chorev and Goodman reviewed the majority of these examples.[69,70] In this section we briefly cover the significant bioactive PMRI peptide milestones.

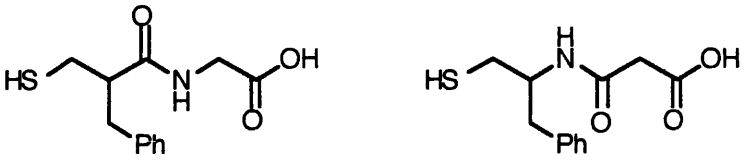
(a) Enkephalin

Chorev, Goodman and co-workers synthesised the first highly bioactive PMRI peptides, the enkephalin analogues: Tyr-D-Ala-Gly-Pheψ(NHCO)Leu-NH_2, Tyr-D-Ala-Gly-Pheψ(NHCO)Met-NH_2, Tyr-D-Ala-Gly-*g*Phe-D-*r*Leu-For and Tyr-D-Ala-Gly-*g*Phe-D-*r*Met-For.[102] All four analogues displayed higher activity than Met-enkephalin in an *in vitro* test, and prolonged duration of action (*in vitro* and *in vivo*), presumably because the modifications protect the pseudopeptides from enzymatic degradation.[102] The latter two analogues were the more active of the four.[102]

Further work on PMRI enkephalins has met with more qualified success.[69]

(b) Protease inhibition

By applying the retro-inverso concept to thiorphan, **90**, Roques and co-workers achieved complete differentiation of its inhibitory activity.[166] Thus, whereas thiorphan, **90**, is a highly potent inhibitor of both enkephalinase (K_i = 3.5 nM) and a less potent inhibitor of ACE (K_i = 140 nM), "retro-thiorphan", **91**, inhibits enkephalinase with great selectivity [K_i = 6 nM vs IC_{50} (against ACE) > 10,000 nM].[166]



90 **91**

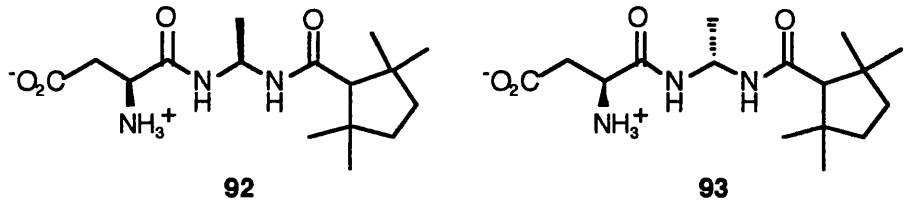
Figure 11: Thiorphan, **90** and retro-thiophan, **91**.

Retro-thiorphan, **91**, and thiorphan, **90**, similarly affected analgesia (due to protection of endogenous enkephalins) in *in vivo* studies.[166] Crystal structures of (*S*)-thiorphan, **90**, and (*R*)-retro-thiorphan, **91**, bound to thermolysin (which shares many active site residues with enkephalinase) show that the two inhibitors utilise very similar interactions with the enzyme (including the hydrogen bonding to the amide linkage).[167]

Roques and co-workers applied the same approach, and further modifications to the zinc metallopeptidase inhibitor kelatorphan, to similar effect.[168,169]

(c) Sweeteners

As part of their on-going investigation of the structure - taste relationship of peptides and peptidomimetics,[170] Goodman and co-workers synthesised the retro-inverso peptide sweeteners (based on the Asp-D-Ala-NHR motif), **92** and **93**, depicted in figure 12.[171-173]



92 **93**

Figure 12: Retro-inverso sweeteners.

These pseudopeptides, **92** and **93**, are *ca.* 800 times sweeter then sucrose.[171]

(d) Gastrin antagonism

Martinez and co-workers produced potent gastrin antagonists by partial retro-inverso modification of its carboxy terminal tetrapeptide, Trp-Met-Asp-Phe-NH_2.[174] The analogue Boc-Trp-Leu-*g*Asp-$CO(CH_2)_2$Ph displayed *in vivo* gastrin antagonism (and no agonism) with enhanced duration of action.[174]

(e) CCK

Following on from their work on gastrin, Martinez and co-workers applied the PMRI approach to CCK-7 and 8, (Asp-)Tyr(SO_3^-)-Met-Gly-Trp-Met-Asp-Phe-NH_2.[124] Roques and co-workers also synthesised PMRI CCK-7 analogues.[175] Both groups reversed the Met^{28}-Gly^{29} bond (a major cleavage site), replaced both Met^{28} and Met^{31} with Nle, and used amino terminal Boc protection (the latter two modifications being precedented[176]). All of these PMRI CCK-7 and 8 analogues exhibited affinity to the CNS CCK receptor, much lower affinity to the peripheral CCK receptor, and low functional potency. Martinez and co-workers' analogue with highest affinity to the CNS receptor, Boc-Asp-Tyr(SO_3^-)-Nleψ(NHCO)Gly-Trp-Nle-Asp-Phe-NH_2, displayed lower affinity than Boc[$Nle^{28,31}$]CCK-7,[124] whereas Roques and co-workers' analogue, Boc-Tyr(SO_3^-)-Nleψ(NHCO)Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH_2, displayed higher affinity and high resistance to proteolysis.[175]

(f) Tuftsin

Tuftsin, Thr-Lys-Pro-Arg, is degraded *in vivo*, principally between Thr and Lys, to produce tripeptides which inhibit its activity. Therefore Verdini and co-workers synthesised the PMRI tuftsin, Thrψ(NHCO)Lys-Pro-Arg, (part of their synthesis is shown in scheme 22).[146] This PMRI tuftsin displayed prolonged *in vitro* stability in human plasma (less than 2% hydrolysis after 50 m *c.f.* complete hydrolysis of tuftsin in less than 8 m).[146] It does, however, suffer hydrolysis to H_2N-*m*Lys-Pro-Arg [in accordance with the usual path of decomposition of *gem*-diaminoalkyl compounds, see section **1.5.1***(a)(i)(5)*].[146]

Three types of *in vivo* test (including oral administration) demonstrated that the PMRI tuftsin enhanced immune responses more effectively than tuftsin itself.[146]

Subsequent tests have further demonstrated the higher immunostimulatory activity of this PMRI tuftsin compared with natural tuftsin.[177,178] The enhancement of activity is thought to be due to the PMRI tuftsin's resistance to peptidases and the fact that its hydrolysis product does not interfere with its activity.[146]

FAB tandem mass spectrometry revealed that, despite the stability described above, the PMRI tuftsin is labile under FAB conditions with the most abundant fragmentation processes involving the *gem*-diamino group.[179] Indeed, it is generally the case for PMRI peptides that ions formed by fragmentation in the vicinity of the *gem*-diamino residue are particularly abundant, which may be interpreted as indicating lower bond strengths in this region.[180]

(g) The renaissance of retro-inverso and end group modified retro-inverso peptides

Chorev and Goodman reviewed the recent resurgence of retro-inverso and end group modified retro-inverso (*i.e.* not partially modified retro-inverso) peptides in the areas of combinatorial chemistry and immunology,[70] where end group modified retro-inverso antigens show great promise for the development of vaccines, immunomodulators and immunodiagnostics.[181]

(h) Taking the concept beyond pseudopeptides

We have seen how the PMRI peptide concept developed from end group modified retro-inverso and linear retro-inverso peptides, which in turn developed from cyclic retro-inverso peptides. The PMRI peptide concept constituted the arrival of the retro-peptide bond as a true peptide bond surrogate, which has been widely applied in various biologically relevant peptides. The retro concept has recently been applied to amide bonds other than those in simple peptides, *i.e.* in peptide nucleic acids (PNA)[182,183] and sugar - amino acid links.[184]

1.5.3 *Conformational implications of partial retro-inverso modification.*

(a) The amide bond itself

The retro amide bond obviously scores highly in similarity tests against the natural peptide bond,[38,69,185] indeed, with some protocols it scores perfectly, due to symmetry.[57]

However, in terms of bond dimensions and permitting identical conformers to be adopted, the *trans*-alkene surrogate [ψ(*E*-CH=CH)] mimics the natural peptide bond better than does the retro amide bond.[38]

(b) The new residues

As discussed in section **1.5**, partial retro-inverso modification generates two new residues, a *gem*-diaminoalkyl residue and a C^2 substituted malonyl residue. Their presence in a PMRI peptide exerts a profound influence upon its conformation, especially in their immediate vicinity.

Dauber-Osguthorpe and co-workers carried out a complete search of the conformational space available to the *gem*-diamino and malonyl residues derived by partial retro-inverso modification of Ac-Ala-NHMe (*i.e.* Ac-*g*Ala-Ac and MeNH-*m*Ala-NHMe) using a valence force field method.[186] The resultant Ramachandran maps[3,187] (which were similar to the previous results of Stern *et al.*[188,189]) were compared with that of the parent residue.[186] For both PMRI residues the C_7 regions were destabilised (C_7^{eq}, or the inverse γ-turn, being the lowest energy conformation of Ac-Ala-NHMe), due to the proximity of atoms with like charges.[186] The *gem*-diamino residue had its minimum energy in an extended conformation, and also local minima in equivalent helical conformations containing six-membered hydrogen bonded rings (a C_6 arrangement).[186] The malonyl residue had its minimum energy in equivalent helical conformations (C_6 arrangement) and low energy regions corresponding to extended conformations.[186] The reported crystal structures of appropriate *gem*-diaminoalkyl and malonyl residues are compatible with these results.‡

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‡ Of the *gem*-diaminoalkyl residues one is in a helical conformation[190] and seven are in C_7^{eq} / extended conformations.[173,191-196] Of the malonyl residues five (all -*m*Gly-) are in helical conformations[197-200] and three in the C_7^{eq} / extended region.[199,201,202] For a brief survey of model pseudopeptide crystal structures (including most PMRI examples) see reference 185.

In all the discussions of Ramachandran maps herein, the following terms are used, rather loosely, to describe the given, general regions of the maps:

helical - the lower left and upper right hand quadrants [in the general vicinity of the α-helical conformation, *ca.* ±(60°,60°)];[3]

extended - the upper left and lower right hand corners;

From these results were deduced the implications of the incorporation of the modified residues into secondary structures, based on the reasoning that "a residue will be likely to be part of a secondary structure element if the required conformation corresponds to a local minimum of the energy surface of the isolated residue. Conversely, if a specific conformation is very unstable for an isolated residue it is not likely to be observed in this conformation."[186] Inter-residue interactions were also considered.[186]

(i) β-turns

Four residues are involved in a β-turn, with a hydrogen bond between the carbonyl of residue i and the NH of residue i + 3. The six types of ideal β-turn (types I, II, and III, and their mirror images) differ in the (ϕ,ψ) angles of residues i + 1 and i + 2.[3,187,203,204] If the first amide bond of a β-turn is reversed, then so must be the third in order to enable the i ... i + 3 hydrogen bond to form.[186] The compound modelled of this type [Acψ(NHCO)Ala-Alaψ(NHCO)NHMe] formed no stable β-turns because the *gem*-diaminoalkyl residue has a very high energy in the conformations necessary for the i + 2 position.[186]

Reversal of the second amide bond [model compound Ac-Alaψ(NHCO)Ala-NHMe] resulted in a PMRI peptide that could form a stable type II β-turn, although an extended conformation was more stable.[186]

These predictions are supported by experimental studies. ^{1}H NMR and IR spectroscopic studies in solution of Bu^{t}CO-Ala-Gly-NHPr^i and its three PMRI analogues demonstrated that reversal of the middle amide bond exerts little influence on the propensity to form β-turns, in stark contrast to reversal of the other two amide bonds.[195] However, the crystal structure of Bu^{t}CO-Alaψ(NHCO)Gly-NHPr^i was "quasi-extended" with no intramolecular (only intermolecular) hydrogen bonding, whereas the parent peptide adopted a type II β-turn.[195] Intermolecular interactions in the crystal are presumably responsible for this difference.

For a 9-membered hydrogen bonded ring conformation specific to PMRI peptides that resembles a native β-turn see section **1.5.3***(b)(iv)(2).*

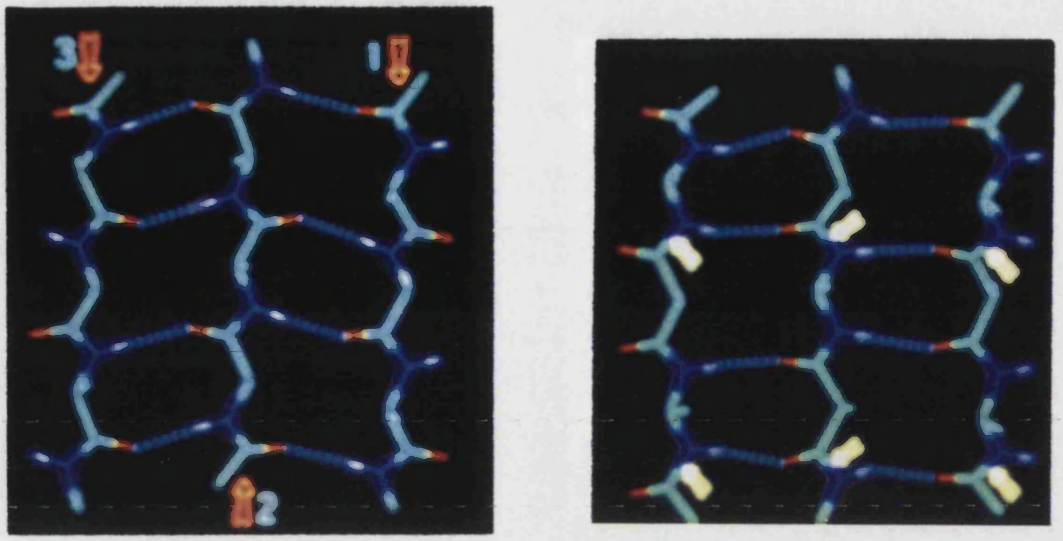
β-sheet region - the upper left hand corner, near the protein β-sheet conformation, *ca.* (-130°,120°)].[187]

(ii) α-helices

An α-helix has a regular hydrogen bond network between the carbonyl of residue i and the NH of residue i + 4, with all dipoles aligned.[3,187,204] Reversal of one amide bond disrupts this hydrogen bond network, and brings two NHs and two carbonyls close together, considerably destabilising the helix.[186] Reversing every third amide bond {model compound: Ac-[ψ(NHCO)Ala_3]$_3$ψ(NHCO)NHMe} produced a helix with a complete hydrogen bond network similar to an α-helix.[186] But it was not as stable as a native α-helix because the dipole alignment is less favourable and the *gem*-diaminoalkyl residues are not in their lowest energy conformation.[186] No experimental studies applicable to such systems have been performed.

(iii) β-sheets

β-Sheets consist of parallel or antiparallel extended peptide chains hydrogen bonded to each other.[3,187,204] The smallest unit of a β-sheet is not one strand (*i.e.* one extended peptide chain) but two hydrogen bonded strands, since the hydrogen bonds go from one strand to another.[204] Dauber-Osguthorpe and co-workers used three strands to construct model parallel and antiparallel β-sheets and calculated the energy of sheet formation with respect to the isolated extended strands [the model antiparallel β-sheets are depicted in figure 13]. In common with α-helices, indiscriminate amide bond reversal results in disruption of the β-sheet's hydrogen bond network and hence destabilsation. But by reversing every second amide bond [model strands: Ac-Alaψ(NHCO)Ala-Alaψ(NHCO)NHMe and Acψ(NHCO)Ala-Alaψ(NHCO)Ala-NHMe] fully hydrogen bonded parallel and antiparallel β-sheets were constructed.[186] Unlike natural β-sheets, these modified sheets have all their carbonyls pointing one way, and all their NHs pointing the other [see figure 13(b)], which is a similarly favourable arrangement to that of a native α-helix and results in strong interstrand attraction.[186] Thus, stable parallel and antiparallel β-sheets were formed, which each had a significantly more favourable energy of formation than the native β-sheets in Dauber-Osguthorpe and co-workers' study.[186]



(a) (b)

Figure 13: Model anti-parallel β-sheets: (a) native peptide; and (b) PMRI peptide with every second amide bond reversed (retro amide bonds indicated by arrows). Only heavy atoms and amide hydrogens shown. Hydrogen bonds indicated by dotted lines.[186]

(1) An experimental investigation

This project aimed to study the influence of retro-amide bonds on PMRI peptide conformation. In particular, we aimed to investigate the effects of systematic, multiple reversals in one molecule, and the propensity of the resultant PMRI peptide to form secondary structure. Founded on the theoretical study of Dauber-Osguthorpe and co-workers (described above),[186] this project aimed specifically to investigate the PMRI peptide motif with every second amide bond reversed, *i.e.* $[Xaa\psi(NHCO)Yaa]_n$ with $n \geq 2$. According to Dauber-Osguthorpe and co-workers' study, such PMRI peptides are expected to be able to adopt β-sheet type structures [see *(iii)*, above].[186]

Prior experimental studies applicable to this motif are somewhat limited. El Masdouri *et al.* described the model PMRI peptides Bu^tCO-*g*Val-$COBu^t$ and MeNH-*m*Val-NHMe, in their respective crystals, as "hydrogen bonded in such a way as to form a parallel β-sheet structure."[196,201] But the hydrogen bonded structures in both these cases are 12-membered rings, rather than the 10-membered rings found in Dauber-Osguthorpe and co-workers' model PMRI β-sheets [figure 13(b)], because El Masdouri *et al.*'s crystallised model peptides do not contain both *gem*-diamino and malonyl residues.

To the best of our knowledge the only investigation of the stability of these 10-membered hydrogen bonded rings formed between *gem*-diamino and malonyl residues is that of Gardner and Gellman,[205] described in section **1.5.3***(b)(iv)(2)*.

Therefore this project aimed to synthesise and study the conformational behaviour of PMRI peptides containing the motif $[Xaa\psi(NHCO)Yaa]_n$, $n \geq 2$.§

The one literature example of a PMRI peptide containing this motif is Argψ(NHCO)(*R,S*)Lys-Aspψ(NHCO)(*R,S*)Val-Tyr, a biologically active PMRI analogue of the immunomodulatory peptide thymopentin.[206] However the authors made no specific mention of its synthesis or conformation.

For this study it was obviously necessary to make specific choices for residues Xaa and Yaa. We chose Yaa = Gly, in order to avoid complications caused by the configurational lability of malonyl residues [see section **1.5.1***(a)(ii)(7)*] and Xaa = Gly (for simplicity) or Ala {the simplest possible side chain, and the residue used in Dauber-Osguthorpe and co-workers' modelling study [see section **1.5.3***(b)*, above]}. These residue changes, with respect to Dauber-Osguthorpe and co-workers' modelling study, will exert some conformational influence. Comparison of previously reported experimental results and the modelling studies of both Stern *et al.* and Alemán and co-workers [section **1.5.3***(b)(iv)(1)*] allow an estimate of these to be made.

The Ramachandran maps calculated by Stern *et al.* for the two model compounds MeNH-*m*Xaa-NHMe, Xaa = Gly or Ala, are very similar,[188] indicating that the presence of a side chain little affects the conformational preference of a malonyl residue. However, the region of the maps of particular interest in this project, that corresponding to the β-sheet conformation, is that which most differs between the studies of Stern *et al.*[188] and Dauber-Osguthorpe and co-workers.[186] In Stern *et al.*'s study, especially for Xaa = Gly, the minima in this region are very shallow and ill defined.[188] It is therefore difficult to

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§ Throughout this thesis the compounds studied are described as PMRI peptides. In fact the "I" is superfluous because they contain no inverted chiral centres, nor any that require inversion in order to maintain topochemical complimentarity with a natural peptide. Nevertheless the "I" is retained because the absence of such centres means it is not incorrect, and its retention avoids the generation of further nomenclature in an already overburdened field.

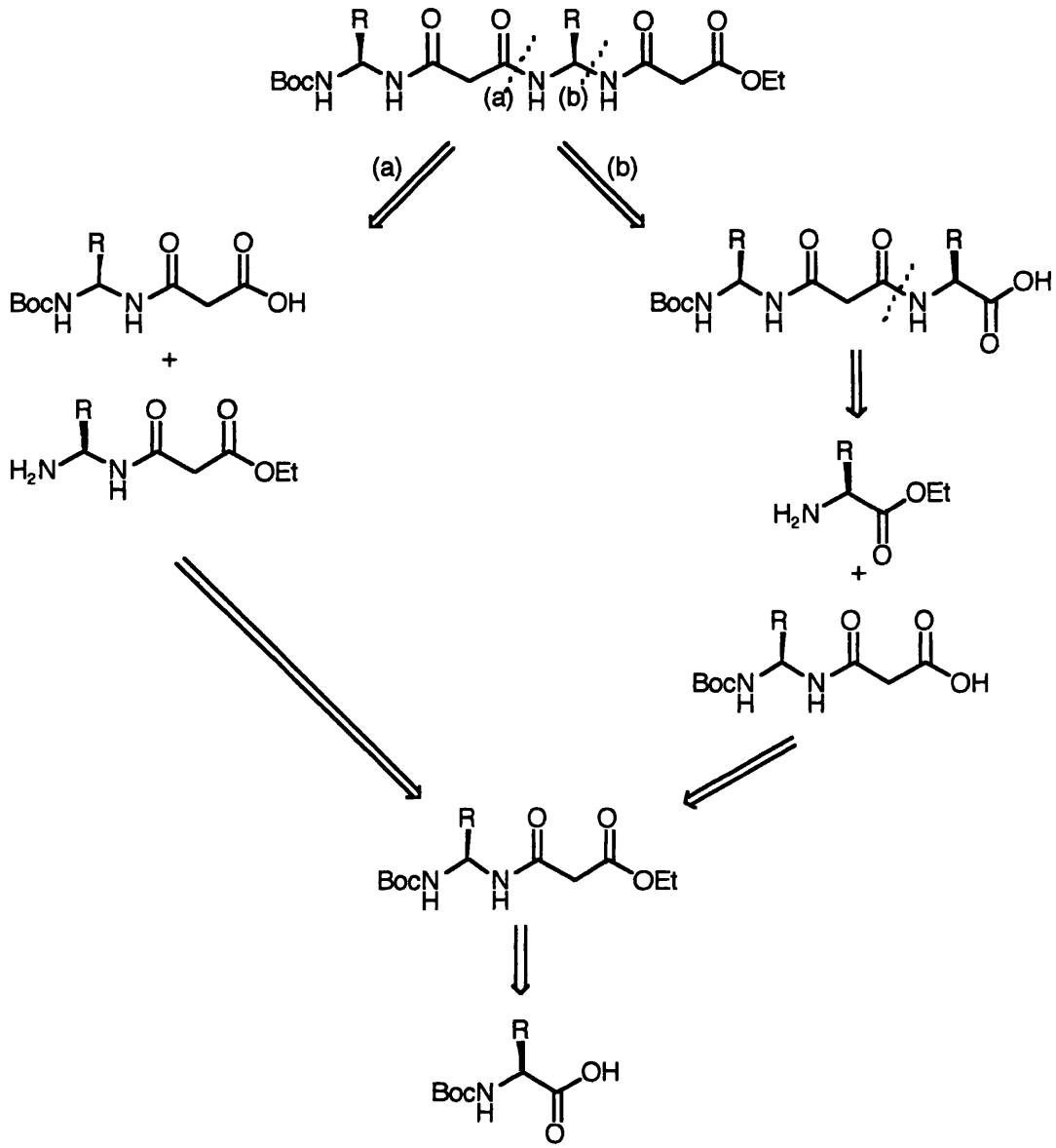
deduce the effect of a side chain on the propensity of malonyl residues to adopt a β-sheet type conformation. The available crystal structures suggest that the absence of a side chain makes an extended conformation less likely: those malonyl residues in extended conformations in the crystalline state all possess side chains.[199,201,202]

The Ramachandran maps calculated by Stern *et al.* for the two model compounds Ac-*g*Xaa-Ac, Xaa = Gly or Ala, display differences: there are only minima in the extended region for Xaa = Ala.[188] Therefore the absence of a side chain in a *gem*-diamino residue disfavours an extended conformation with respect to a helical conformation. El Masdouri *et al.*'s crystal structures of Bu^{t}CO-*g*Val-COBu^t (extended)[196] and Ac-*g*Gly-Ac (helical)[207] support this view.

Therefore of our target motifs, [Xaaψ(NHCO)Gly]$_n$ ($n \geq 2$), Xaa = Ala is expected to be more likely to adopt a β-sheet type structure than Xaa = Gly. Our initial targets were the simplest, blocked examples of this motif, *i.e.* n = 2. We chose Boc and OEt as the *N*- and *C*- terminal protecting groups respectively. This choice was made in the light of the previous success in synthesising PMRI peptides bearing these protecting groups (see section **1.5.1**) and in order to investigate *N*-Boc deprotection methods that would avoid the previously described decomposition problem [see section **1.5.1***(a)(i)(1)*].

Thus the initial targets were Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, and Boc-Alaψ(NHCO)Gly-Alaψ(NHCO)Gly-OEt, which were accessible by coupling two PMRI dipeptide moieties [route (a), scheme 39] or disconnection to a PMRI tripeptide and ultimately a PMRI dipeptide [route (b), scheme 39]. For the PMRI dipeptides we planned to use the Goldschmidt and Wick type synthesis, previously developed in this department [see section **1.5.1***(a)(i)(1)*].

Route (a) was chosen because it is simpler, more symmetrical and only uses the moderate yielding Goldschmidt and Wick type reaction once [for the synthesis of the PMRI dipeptide, as opposed to route (b), which necessitates a second Goldschmidt and Wick type reaction to proceed from the penultimate PMRI tripeptide to the target PMRI tetrapeptide].



Scheme 39: Retrosynthesis of the target PMRI tetrapeptides (*e.g.* **104**: R = H).

Further targets were planned: PG^1-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-PG^2 (*e.g.* **140**: Xaa = Gly, PG^1 = Boc and PG^2 = OEt; or **146**: Xaa = Gly, PG^1 = Ac and PG^2 = NH_2). These PMRI decapeptides were designed to incorporate two of the initial target units linked by a Pro-Gly segment, which promotes (type II) β-turn formation.[187,203,204,208-210] The resultant PMRI decapeptide should therefore be able to turn back on itself and form an intramolecularly hydrogen bonded structure analogous to a short length of antiparallel β-sheet, a structure known as a

β-hairpin.[204,211,¶] Thus, unlike the initial target PMRI tetrapeptides (*e.g.* **104**), these ultimate target PMRI decapeptides (*e.g.* **140** and **146**) constitute the smallest possible β-sheet unit containing our target motif (see schemes 71 and 73 for depictions of folded **140** and **146** respectively).

A molecular modelling energy minimisation [using DISCOVER (Biosym)] was performed on Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NHMe (*i.e.* a simple, blocked example of the ultimate target PMRI decapeptides) and snapshots are depicted in figure 14.[213] This study indicated that a twisted β-hairpin structure [figure 14(d)] is likely to be more stable than the ideal β-hairpin structure [figure 14(a)]: unfavourable interactions between the carbonyls of each strand cause twisting, but do not overcome the favourable interstrand hydrogen bonding.

(2) Other β-sheet mimics

Studies of β-sheets using model peptides or peptidomimetics are fewer than those of β-turns or α-helices, largely because of the lack of simple models and because aggregation frequently causes insurmountable problems.[187,212] Nevertheless, some successful β-sheet peptidomimetics have been synthesised.[32,34,37] Those featuring modified backbones are of note here, *e.g.* Smith, Hirschmann and co-workers' pyrrolinine-based mimics,[214] which have given rise to HIV-1 protease and renin inhibitors;[50,215] Kemp and co-workers' epindolidione-derived β-sheet templates;[13,28,216] Clardy, Schreiber and co-workers' "vinylogous polypeptides" {*i.e.* containing a [Xaaψ(*E*-CH=CH-CONH)Yaa]$_n$ motif};[217] and Ranganathan and co-workers' range of modified peptides containing core units -CO(CH$_2$)$_n$CO- (n = 0, 2... 6, or 8), or NHCOCO, many of which form β-sheet type structures:[218-221] figure 15.

¶ For examples of model peptide and peptidomimetic containing β-hairpins see references 73, 212 and references therein.

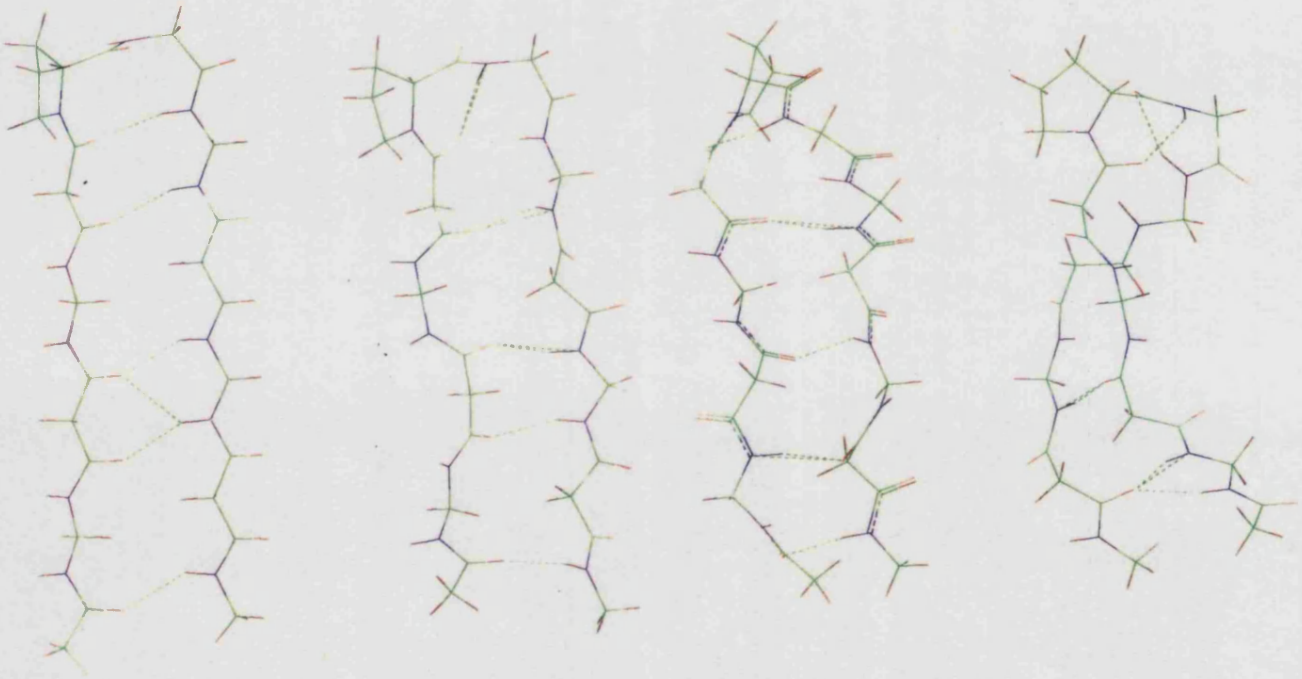


Figure 14: Energy minimisation snapshots of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NHMe. The figures are the relative energies in kcal/mol/residue.[213]

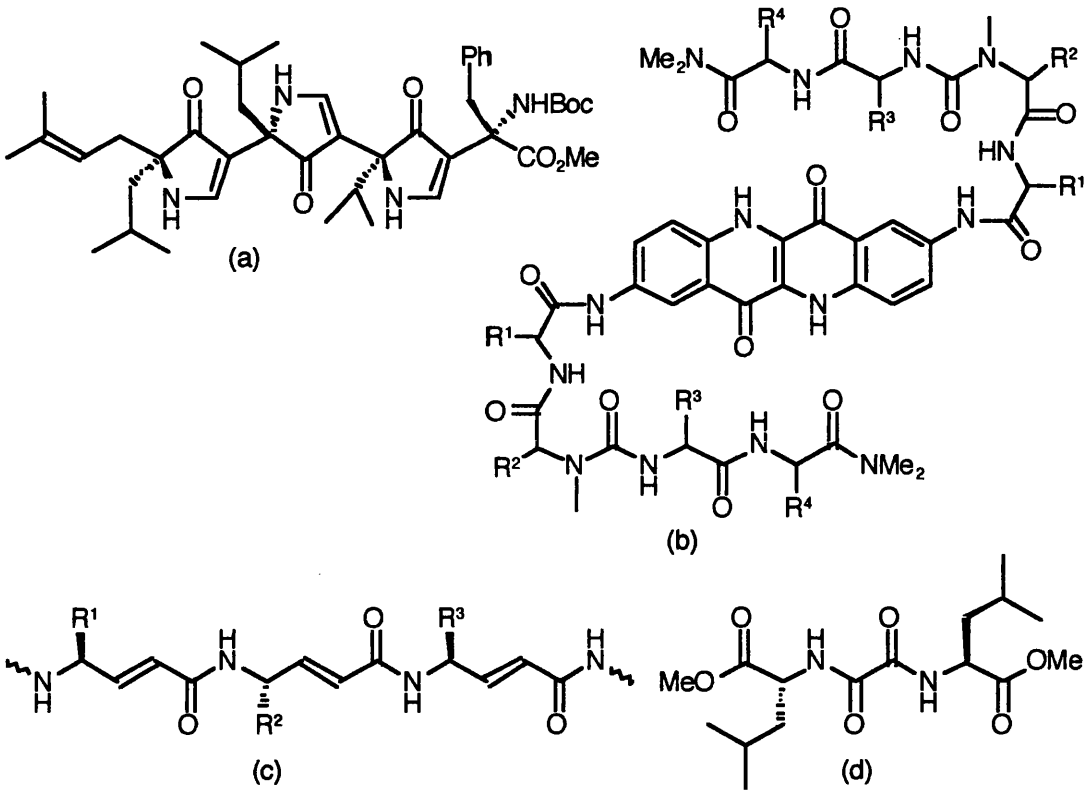


Figure 15: Examples of β-sheet peptidomimetics featuring modified backbones. (a) A pyrrolinine-based mimic;[214] (b) an (epindolidione-derived) templated β-sheet;[216] (c) the "vinylogous polypeptide" backbone;[217] and (d) the "oxalo-retro-peptide" MeO-*r*Leu-COCO-Leu-OMe.[219,*]

(iv) Other conformational studies of PMRI peptides

Experimental and modelling studies have been performed on various PMRI peptides of biological significance,[69,70] but these, not being model PMRI peptides, permit few further general conclusions to be drawn.

However two other groups of studies are generally applicable.

(1) Aléman, Puiggalí and co-workers: the nylon perspective

As mentioned in section **1.5.1***(a)(i)(4)*, nylon 1,3 is identical to $[Gly\psi(NHCO)Gly]_n$, **50**. Thus there is interest in *gem*-diamino residues as components of nylons 1,n; and malonyl residues as components of nylons n,3. Aléman, Puiggalí and co-workers have carried out many theoretical studies on model compounds containing *gem*-

* The use of the term "retro" in the context of these modified peptides (that are conceptually similar to PMRI peptides) does not conform exactly with the definition provided in the **Notes on Nomenclature** section and used elsewhere in this thesis.

diamino and malonyl residues, using AM1 SCF-MO (*i.e.* quantum mechanical) calculations (supported by *ab initio* calculations); much of this work is summarised in their recent publication, reference 222. In that study, Aléman, Puiggalí and co-workers built upon their earlier investigations of Me_2N-*m*Gly-NMe_2,[223,224] Me_2N-*m*Ala-NMe_2,[225] Me_2N-*m*Aib-NMe_2,[225] and Ac-*g*Gly-Ac[226] to parameterise a force field suitable for use with *gem*-diamino and malonyl residues. Their motive for this enterprise was the discrepancy between their earlier results and the corresponding force field calculations of Dauber-Osguthorpe and co-workers[186] and Stern *et al.*[188] (discussed above).

Aléman, Puiggalí and co-workers attributed these discrepancies to "deficiencies in the force field parameters, which give a poor description of the conformational properties of the excessive attractive interactions, *i.e.* C=O···HN, or the excessive repulsive interactions, *i.e.* C=O−O=C and NH−HN."[222] But the AM1 method is not without criticism: Dado and Gellman showed, by comparison with their experimental studies [see section **1.5.3***(b)(iv)(2)*], that AM1 overestimates the strength of intramolecular hydrogen bonds.[227]

The discrepancies reveal themselves in the computed Ramachandran maps for *gem*-diamino and malonyl residues: though there is overall similarity between the different researchers' maps, the minima are in different positions and of different energy.

Specifically, the map of Alemán and Pérez for Ac-*g*Gly-Ac only featured minima in the helical regions, with merely a saddle point in the β-sheet / extended region.[226] In the minimum energy helical conformation, Ac-*g*Gly-Ac formed two intramolecular six-membered, hydrogen bonded rings[226] [the helical conformations found by Dauber-Osguthorpe and co-workers and Stern *et al.* form one C_6 ring: see section **1.5.3***(b)*, above]. This conformation changed on inclusion of environmental effects (modelled by the introduction of ammonia and formaldehyde in order to mimic intermolecular hydrogen bonding), but a helical conformation with the intramolecular hydrogen bonds was maintained.[226] These results are supported by experimental studies in that Ac-*g*Gly-Ac in the crystalline state[207] and some nylons 1,n[228,229] adopt similar helical conformations;

but no intramolecular hydrogen bonds were detected for Ac-*g*Gly-Ac in solution [see section **1.5.3***(b)(iv)(2)*] or the solid state.[207]

Alemán and Pérez's maps for MeHN-*m*Gly-NHMe[223] and MeHN-D-*m*Ala-NHMe[225] resemble each other and feature four minima in the helical region [different to Stern *et al.*'s helical conformations but similar to those of Dauber Osguthorpe and co-workers, and thus similarly supported by crystal structures: see section **1.5.3***(b)*, above]. The map for MeHN-*m*Gly-NHMe contains no other minima,[223] but that for MeHN-D-*m*Ala-NHMe has an additional local minimum in a more extended conformation.[225] In the helical conformations the molecules form a six-membered, hydrogen bonded ring [also found by Stern *et al.* and Dauber Osguthorpe and co-workers: see section **1.5.3***(b)*, above], and detected experimentally by Gellman and co-workers [see section **1.5.3***(b)(iv)(2)*]. Alemán and Pérez found that their predicted minimum energy conformation of MeHN-*m*Gly-NHMe is changed by hydration, but remains helical.[224]

Alemán and Puiggalí computed Ramachandran maps for Ac-*g*Ala-Ac and Ac-*g*Aib-Ac using the AM1 method and found reasonable agreement between them and the conformational preferences obtained using their newly parameterised force field.[222] The map for Ac-*g*Ala-Ac is broadly similar to their map for Ac-*g*Gly-Ac, previously calculated (discussed above). However, in addition to helical minimum energy conformations, Ac-*g*Ala-Ac was also found to posses semi-extended minima [different from those found by Dauber-Osguthorpe and co-workers and Stern *et al.* for Ac-*g*Ala-Ac: see section **1.5.3***(b)*, above].[222] Alemán and Puiggalí further used their force field to model nylons 1,3 and 1,5 and found good correlation with the X-ray data,[139,230] *i.e.* a three-fold helical structure with three hydrogen bond directions and the dihedral angles of the *gem*-diamino residues close to those found for the model compounds; as opposed to the more commonly observed (for nylons m,n where m or n are odd) γ structure, which resembles a protein β-sheet.[228,229]

Alemán and co-workers have further studied the PMRI peptides Ac-Glyψ(NHCO)Gly-NHMe,[231] and Ac-Gly-Glyψ(NHCO)Gly-NHMe,[232] as nylon models, using AM1 and force field methods. Ac-Glyψ(NHCO)Gly-NHMe was found to posses helical minima, some resulting in C_6 hydrogen bonded rings involving the -*m*Gly^2-

residue carbonyls[231] [similar to those found by Dauber-Osguthorpe and co-workers: see section **1.5.3***(b)*, above]. When applied to an infinite nylon 1,3 chain model, results consistent with previous models of the crystal structure were obtained.[231] Only the application of an unfavourable symmetry constraint produced minima in an extended / β-sheet conformation for Ac-Glyψ(NHCO)Gly-NHMe.[231] Ac-Gly-Glyψ(NHCO)Gly-NHMe was computed to be rather flexible, but with a strong tendency to fold and adopt conformations featuring intramolecular hydrogen bonds.[232] An infinite chain model produced two low energy conformations: an α-helix [as previously described by Dauber-Osguthorpe and co-workers for the corresponding alanine model sequence: see section **1.5.3***(b)(ii)*], and a six-fold helix, stabilised by intra and intermolecular hydrogen bonds respectively.[232] A model β-sheet structure was of higher energy because of unfavourably close contacts between C=O•••O=C and NH•••HN.[232]

The conformational predictions of Alemán, Puiggalí and co-workers of relevance to this project may be summarised as indicating that helical structures are always of lower energy than extended or β-sheet type structures for the *gem*-diamino and malonyl residue containing model compounds that they studied, irrespective of the nature of the side chain. This contrasts with the predictions of Dauber-Osguthorpe and co-workers [see section **1.5.3***(b)*, above]. However, since Alemán, Puiggalí and co-workers are primarily interested in nylons (*i.e.* infinite chains) their modelling studies do not provide any obvious structures to seek out in our target PMRI peptides, in order to experimentally confirm or refute their predictions.

(2) Gellman and co-workers: small molecules

Gellman and co-workers have conducted extensive studies on the conformation directing effects of noncovalent interactions, in particular hydrogen bonds, in small molecules in order to deduce their impact on biopolymer folding. In the course of these studies they have investigated some small molecules containing a malonyl residue, which are of interest in the PMRI peptide context.

Gellman and co-workers' results from VT IR and NMR spectroscopy (in dichloromethane and acetonitrile) and X-ray crystallography imply that the 9-membered

hydrogen bonded ring conformation, depicted in figure 16, is intrinsically favourable to the *N*-malonylglycine or alanine unit.[29,233-236]

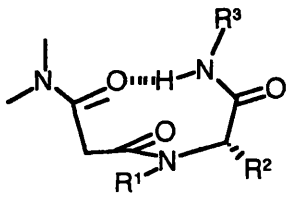


Figure 16: The favoured conformation of *N*-malonylglycine (R^2 = H) or alanine (R^2 = Me). R^1 = H, Me, or Et; R^3 = Me, Bn, 1-adamantyl.

Gellman and co-workers therefore asserted that, "the malonyl-*N*-methyl-amino acid subunit... represents a potential alternative to the natural dipeptide subunit at residues i + 1 and i + 2 of a β-turn."[234]

This folding pattern could possibly be adopted by our ultimate target PMRI decapeptides (*e.g.* **140** and **146**), across residues -*m*Gly^4-Pro^5-Gly^6-.

Gellman and co-workers also demonstrated, using VT IR and ^{1}H NMR spectroscopy, that *N,N,N'*-trimethylmalonamide adopts a six-membered hydrogen bonded ring conformation in dichloromethane solution, and less so in acetonitrile.[237] A similar hydrogen bonded ring is found in the crystal structures of some malonamide derivatives.[235] The detection of the hydrogen bond concurs with the predictions of Stern *et al*, Dauber-Osguthorpe and co-workers, and Alemán and Pérez [*c.f.* section **1.5.3***(b)*, above].

Jorgensen and co-workers explained the variations observed among association constants for a series of triply hydrogen bonded complexes (pertinent to nucleotide base-pairing) by considering the "secondary interactions" among the hydrogen bonded groups, *i.e.* electrostatic interactions between donor and acceptor atoms forced to approach each other due to the formation of a primary hydrogen bond.[238,239] Moreover they went on to predict that secondary interactions would lead to greater stability for the PMRI dipeptide dimer depicted in figure 17(b), than for the corresponding glycine dimer, figure 17(a), when the hydrogen bonding groups were constrained to be planar.[238]

Gardner and Gellman published an experimental test (using IR spectroscopy in dichloromethane solution) of the latter prediction, after the work described in this thesis

was completed.[205] The model pseudopeptide for their study was Me_2N-*m*Gly-Pro$\psi(CO_2)$Gly-*g*Gly-Ac, **94**, which should, according to Jorgensen and co-workers' prediction, fold in a β-hairpin type fashion more readily than the corresponding parent depsipeptide, Ac-Gly-Pro$\psi(CO_2)$Gly-Gly-NMe_2. However, Gardner and Gellman found that their IR spectroscopy data, while consistent with the two state equilibrium depicted in figure 18, yielded indistinguishable equilibrium constants for the parent and PMRI depsipeptides ($K \approx 0.6$, in dichloromethane).[205]

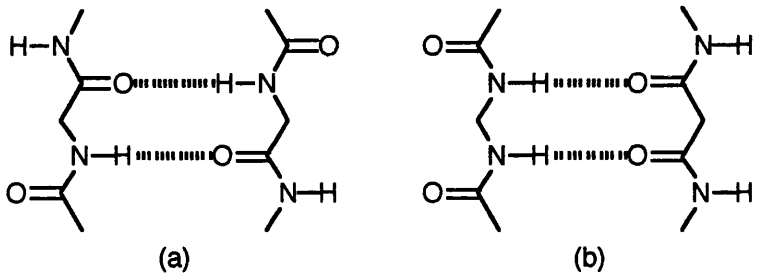


Figure 17: 10-Membered ring hydrogen bonded dimers of (a) blocked glycine, and (b) Ac-*g*Gly-Ac and MeNH-*m*Gly-NHMe.

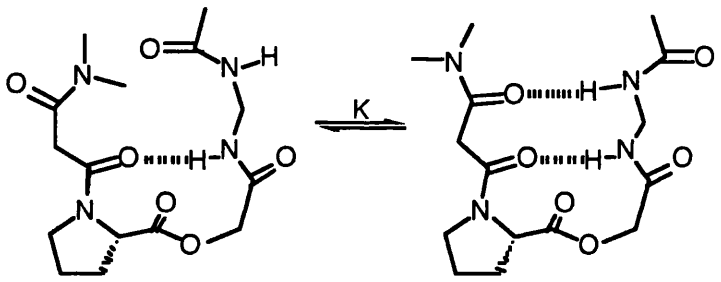


Figure 18: The two state equilibrium found for Me_2N-*m*Gly-Pro$\psi(CO_2)$Gly-*g*Gly-Ac, **94**, [and Ac-Gly-Pro$\psi(CO_2)$Gly-Gly-NMe_2, not shown].

Therefore secondary interactions do not increase the β-folding propensity of **94** over that of its parent, in this case, probably because intramolecular dipole-dipole repulsions result in nonplanarity of the amide groups,[205] a situation that cannot arise within the rigid heterocyclic systems found in nucleotide bases.

In the course of their study, Gardner and Gellman also demonstrated that Ac-*g*Gly-Ac exhibits no intramolecular hydrogen bonding in dichloromethane solution.[73] This result accords with Dauber-Osguthorpe and co-workers' modelling study, which predicted that a six-membered hydrogen bonded ring arrangement of Ac-*g*Ala-Ac is less stable than the extended conformation; but it contrasts with the predictions of Alemán and Pérez, and

Stern *et al.* that such an arrangement is the minimum energy conformation for Ac-*g*Gly-Ac [section **1.5.3***(b)*, above].

To the best of our knowledge this study by Gardner and Gellman is the only previous investigation of the 10-membered hydrogen bonded rings found in PMRI peptide β-sheets [see section **1.5.3***(b)(iii)*].

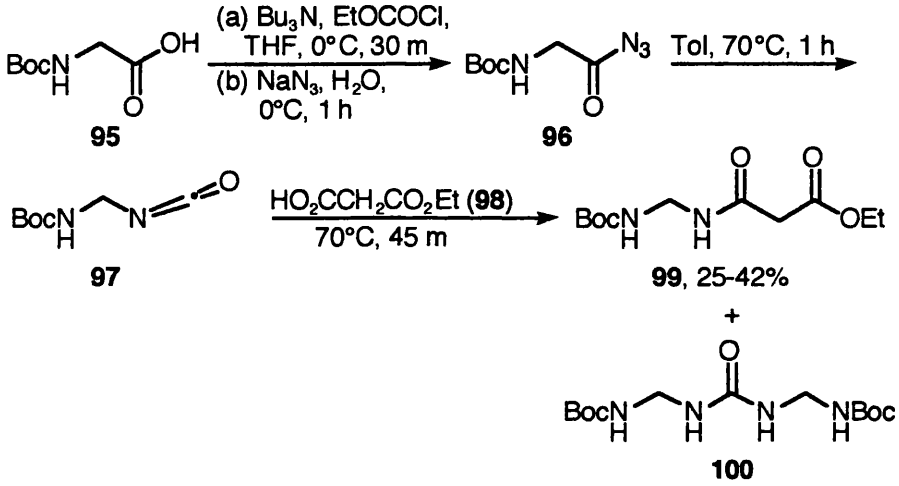
Chapter Two: Results and Discussion

2.1 *Synthesis of Protected PMRI Tetrapeptides, Boc-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-OEt, **104**, **122** and **126**.-*

2.1.1 *Synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**.*

*(a) Synthesis of Boc-Glyψ(NHCO)Gly-OEt, **99***

The approach to Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, was through the coupling of two protected Glyψ(NHCO)Gly moieties. Therefore Boc-Glyψ(NHCO)Gly-OEt, **99**, was synthesised from Boc-glycine, **95**, using the Goldschmidt and Wick type procedure previously developed in this department to synthesise PMRI dipeptides: scheme 40.[84]



Scheme 40: Goldschmidt and Wick type synthesis of Boc-Glyψ(NHCO)Gly-OEt, **99**.

Boc-glycine, **95**, was converted to the acyl azide, **96**, via its mixed anhydride, and the acyl azide, **96**, was extracted from the reaction mixture. It underwent the Curtius rearrangement on heating affording the isocyanate, **97**, which with monoethyl malonate, **98** (obtained from benzyl ethyl malonate by hydrogenolysis), gave Boc-Glyψ(NHCO)Gly-OEt, **99**, in 25% yield.

In the light of the yields of 60-75% obtained previously for the PMRI dipeptides, R^1-Xaaψ(NHCO)Gly-OR^2 (R^1 = Z, Boc, Ac; R^2 = Et, Ph; Xaa = Ala, Val, Phe), using this method,[84] the yield of 25% for Boc-Glyψ(NHCO)Gly-OEt, **99**, was disappointing. Therefore an investigation of the reaction by-products was performed. TLC analysis (mixture E) indicated the presence of much Boc-glycine, **95**, (which was not recovered),

prior to the extraction of the acyl azide, **96**: presumably Boc-glycine, **95**, was regenerated by hydrolysis of the mixed anhydride upon addition of the aqueous sodium azide solution. TLC analysis (mixture E) of the crude crystalline product and filtrate indicated that some monoethyl malonate, **98**, remained unreacted (more being in the filtrate than in the solid). The purification of Boc-Glyψ(NHCO)Gly-OEt, **99**, was therefore difficult, a number of recrystallisations being required. Comparison of our results with those of Chorev, Goodman and MacDonald [see scheme 5, section **1.5.1***(a)(i)(1)*] suggested that one of the (low yield) isolated by-products was the urea, **100**, a fact borne out by experimentation; none of the other by-products could be identified.

In order to address these problems, a modified procedure was devised. In the new procedure only 1 eq of monoethyl malonate, **98**, was used (rather than 1.2 eq in the original procedure) and the work-up was altered (see experimental chapter). These changes improved the yield to 42%, a fact attributable to greater ease of purification, and reduced by-product formation {possibly in accordance with Chorev, Goodman and MacDonald's argument relating yield of by-products with reaction mixture polarity [see table 2, section **1.5.1***(a)(i)(1)*][105]}.*

(b) N-*Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99***

(i) Deprotection using hydrogen chloride

Boc-Glyψ(NHCO)Gly-OEt, **99**, was deprotected at its amino terminus using both hydrogen chloride and TFA. Initially deprotection using ethanolic hydrogen chloride was favoured because the reaction appeared to provide the desired product, HCl.Glyψ(NHCO)Gly-OEt, **101**, as a hygroscopic colourless solid, of reasonable purity, in quantitative yield: scheme 41. HCl.Glyψ(NHCO)Gly-OEt, **101**, could be purified, albeit with low recovery, by crystallisation: Furthermore, the work-up procedure was easier than with TFA deprotection (see experimental chapter).

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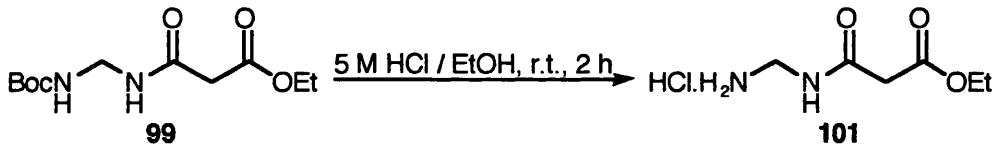
* Other methods of PMRI dipeptide synthesis were investigated for Boc-Valψ(NHCO)Gly-OEt, **116(a)**, see section **2.1.2***(a)*.



99 **101**

Scheme 41: *N*-Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, using ethanolic hydrogen chloride.

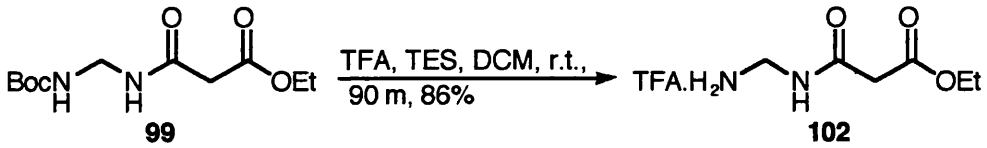
This good result was somewhat surprising given the earlier finding that *N*-Boc deprotection by acid hydrolysis leads to decomposition.[84] However, further experiments revealed that decomposition did indeed occur. The initial deprotection reactions yielded products, the crude ^{1}H NMR spectra of which contained a 1:1:1 triplet at δ_H 7.4 (see figure A1) which was mistakenly disregarded as due to either a minor impurity or ^{14}N - ^{1}H coupling of Gly$^1NH_3^+$ under the acidic conditions. This signal was absent in the ^{1}H NMR spectrum of the crystallised product. Later deprotection reactions (without significant procedural change) yielded crude products, the ^{1}H NMR spectra of which contained an additional Gly$^1\psi$NH signal, an additional Gly$^2C^\alpha H_2$ signal [hidden by the water signal in figure A1(a) but visible in figure A1(b)-(d) and figure A2], and a broadened Gly$^1C^\alpha H_2$ signal [figure A1(a)]. To determine whether this was merely a *cis* / *trans* phenomenon (*i.e.* slowly interconverting isomers with *cis* / *trans* configurations at the amide bond) variable temperature (VT) (figure A1) and NOESY (figure A2) ^{1}H NMR experiments were performed. The NOESY spectrum was consistent with *cis* / *trans* isomers but coalescence of the suspected isomeric signals was not observed on increasing the temperature during the VT experiment. Therefore no firm conclusion can be drawn on the presence of amide *cis* / *trans* isomers in HCl.Glyψ(NHCO)Gly-OEt, **101**. The NOESY spectrum indicated exchange between the Gly$^1NH_3^+$ signal and the signal at δ_H 7.4, and the VT experiment showed strengthening of the δ_H 7.4 signal with increasing temperature. This result, reinforced by the universal degeneration of the ^{1}H NMR spectrum with increasing temperature (which failed to revert to the original on cooling to r.t.), suggested that the δ_H 7.4 signal was due to ammonium chloride,[240,241] a product of HCl.Glyψ(NHCO)Gly-OEt, **101**, decomposition. Addition of ammonium chloride to the NMR sample after the VT experiment and reaccumulation confirmed this assignment. Thus we may conclude that HCl.Glyψ(NHCO)Gly-OEt, **101**, decomposes under the *N*-

Boc deprotection conditions, to yield ultimately ammonium chloride, formaldehyde and ethyl malonamate, **118**, as noted by Loudon and co-workers: see scheme 19, section **1.5.1***(a)(i)(5)* (for **101**: $R^1 = EtO_2CCH_2$, $R^2 = H$ and $R^3 = H$). Of these decomposition products, only ammonium chloride was detected conclusively (during the VT experiments); the final VT ^1H NMR spectrum [figure A1(f)] being insufficiently clear to allow unambiguous assignment.

Changing the deprotection medium to 4 M hydrogen chloride in 1,4-dioxane failed to alleviate the decomposition problems. Therefore purification by neutralisation of HCl.Glyψ(NHCO)Gly-OEt, **101**, and extraction was attempted. However, extraction of a 5% sodium hydrogen carbonate solution of **101** with ether (as recommended Loudon and DeBons for the isolation of Boc-*g*Gly[115]) or ethyl acetate failed to yield any Glyψ(NHCO)Gly-OEt.

(ii) Deprotection using TFA

TFA is a simple alternative to hydrogen chloride for *N*-Boc removal.[242] A recent protocol, developed by Douglas and co-workers, was adopted.[243] This uses triethylsilane (TES) as a carbocation scavenger, which increases the deprotection yield and decreases the reaction time.[243] Thus, TFA.Glyψ(NHCO)Gly-OEt, **102**, was obtained from Boc-Glyψ(NHCO)Gly-OEt, **99**, as an intractable hygroscopic colourless glassy solid in 86% yield, scheme 42.



99 **102**

Scheme 42: *N*-Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, with TFA / TES.

However, the laborious work-up and intractable nature of TFA.Glyψ(NHCO)Gly-OEt, **102**, favoured *N*-Boc deprotection using ethanolic hydrogen chloride until the difficulties described above were recognised. Hence HCl.Glyψ(NHCO)Gly-OEt, **101**, rather than TFA.Glyψ(NHCO)Gly-OEt, **102**, was used in the majority of the coupling reactions requiring Glyψ(NHCO)Gly-OEt (see below). However, when these difficulties became known, TES was omitted during *N*-Boc deprotection using TFA, to no adverse effect, and

the alternative work-up procedure, developed for TFA.Valψ(NHCO)Gly-OEt, **119**, was adopted [see section **2.1.2***(b)(ii)* and experimental chapter]. These altered conditions furnished TFA.Glyψ(NHCO)Gly-OEt, **102**, in 90% yield [containing only a trace of ammonium trifluoroacetate (indicated by ^{1}H NMR spectroscopy)].

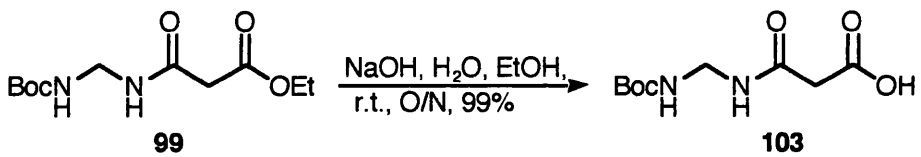
Therefore, although *N*-Boc deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, with hydrogen chloride is simpler practically and the hydrogen chloride salt, **101**, easier to handle, deprotection with TFA is to be recommended, for it results in less decomposition of the product.

(iii) ^{1}H NMR exchange effects

During the course of these *N*-Boc deprotection studies an unexpected NMR exchange effect was encountered. ^{1}H NMR spectra of HCl.Glyψ(NHCO)Gly-OEt, **101**, (and TFA.Glyψ(NHCO)Gly-OEt, **102**) in $DMSO_{d6}$ showed the expected disappearance of the $Gly^1NH_3^+$ signal (δ_H 8.4, figure A1) and broadening of the $Gly^1\Psi NH$ (δ_H 9.2) and $Gly^1C^\alpha H_2$ (δ_H 4.2) signals on D_2O exchange. However, samples run in $methanol_{d4}$ appeared disturbingly simple, containing only the usual signals at δ_H 1.2 and 4.1 (*c.f.* figure A1) and a broad singlet at δ_H 4.4! An experiment with Boc-Glyψ(NHCO)Gly-OEt, **99**, (to avoid any interference due to decomposition) revealed what was happening: a sample of Boc-Glyψ(NHCO)Gly-OEt, **99**, in $DMSO_{d6}$ was left to undergo D_2O exchange for a few days prior to ^{1}H NMR accumulation. The resultant spectrum showed that not only the expected BocNH and $Gly^1\Psi NH$ were exchanged, but also $Gly^2C^\alpha H_2$. This exchange phenomenon proved useful for signal assignment in some subsequent ^{1}H NMR spectra.

*(c) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99***

Saponification of Boc-Glyψ(NHCO)Gly-OEt, **99**, gave the free acid, Boc-Glyψ(NHCO)Gly, **103**, as a colourless solid in 99% yield: scheme 43.



99 **103**

Scheme 43: Saponification of Boc-Glyψ(NHCO)Gly-OEt, **99**.

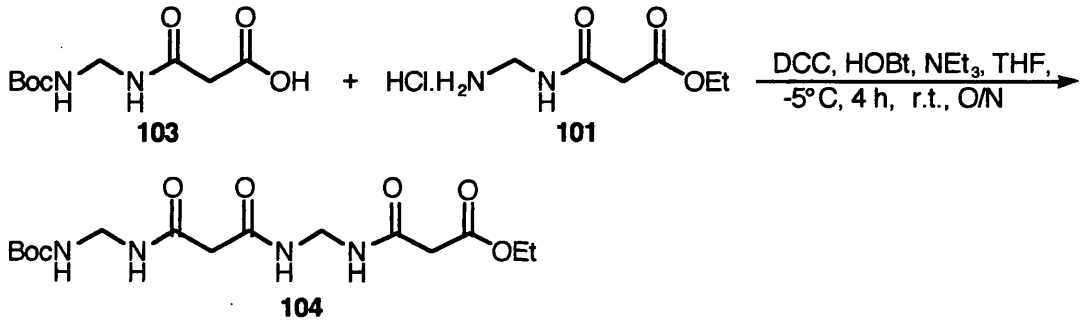
*(d) Coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101***

With the appropriate fragments in hand, the stage was set to couple them to produce the PMRI tetrapeptide Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**.

(i) Carbodiimide reagent mediated coupling

(1) DCC / HOBt

DCC is a well established peptide coupling reagent, successfully employed in PMRI peptide synthesis [see section **1.5.1***(a)(i)(1)*]. Thus Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**, were coupled using the standard DCC / HOBt conditions: scheme 44.[242]



103 **101** **104**

Scheme 44: DCC / HOBt mediated coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**.

Following literature work-up,[242] only a trace of dicyclohexyl urea (DCU) was isolated. The target PMRI tetrapeptide, **104**, was found to have precipitated with DCU and was therefore filtered off during the work-up. Attempts to separate Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** and DCU by extraction, washing, or crystallisation merely served to demonstrate that they display a similar solubility profile (*i.e.* soluble in DMF and DMSO, moderately soluble in methanol and ethanol, and sparingly soluble in many other organic solvents). Column chromatography with neutral alumina as the stationary phase failed.† Therefore column chromatography with silica as the stationary phase, eluting with chloroform / methanol / acetic acid mixtures (of increasing polarity starting

† Thin layer chromatographs on silica plates were visualised using ninhydrin dip solution, followed by heating. This was less successful on alumina (and various reverse phase silica) plates, where iodine and ninhydrin were required, but these still only gave faint spots.

with 95:3:2) was employed, after a qualitative test showed that this solvent system did not deprotect Boc-glycine, **95**. Thus Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, was obtained in 41% crude yield (the sample was contaminated with phthalate plasticiser, presumably accumulated from the large quantities of solvents used in the various purification attempts).

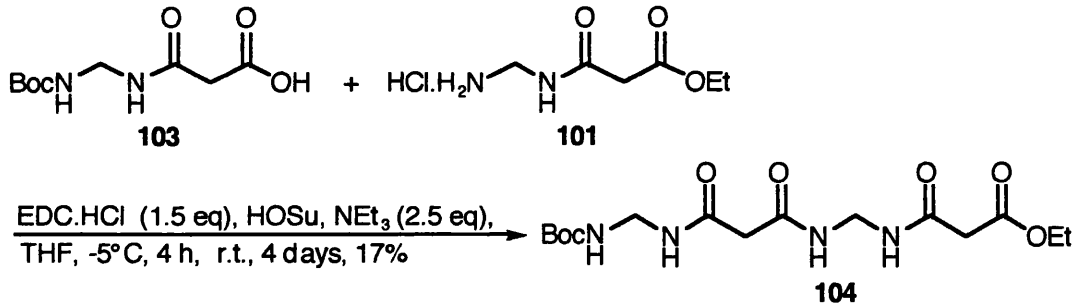
(2) DIPCDI / HOBt

Di-*iso*-propylcarbodiimide (DIPCDI) is a commercially available carbodiimide coupling reagent, the urea by-product [di-*iso*-propyl urea (DIPU)] of which is more soluble in dichloromethane than is DCU.[244] DIPCDI was employed in place of DCC to couple Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**, (otherwise as scheme 44) and produced Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, which could be readily purified and was free from DIPU. The precipitated product was collected and washed sequentially with THF, dichloromethane and water to provide Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, in 18% yield. The low yield is attributed to loss of material during the washings (from which no further product, **104**, could be recovered), a fair quantity of solvent being required to remove the by-products (DIPU is only *ca.* twice as soluble as DCU in dichloromethane[244]).

(3) EDC / HOSu

A third class of carbodiimide coupling reagents are water soluble and produce water soluble ureas.[245,246] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC)[246] is a commercially available member of this class (as the hydrochloride salt) and was used, with HOSu as auxiliary nucleophile, to couple Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**. The change from HOBt to HOSu was made, prompted by the previous syntheses, to aid purification of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**: qualitative experiments indicated that HOSu and **104**, are more easily separated by column chromatography than HOBt and **104**, in addition HOSu is more readily removed than HOBt by washing with water.[247] So Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**, were coupled using excess EDC.HCl [the additional half

equivalent was added after 3 days (slow reactions and / or excess coupling reagent are often found in EDC procedures)[248-250]: scheme 45.



Scheme 45: EDC / HOSu mediated coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**.

The product, **104**, was obtained in 17% yield after work-up and chromatography. Unreacted acid, **103**, was identified by TLC, but not recovered. It was unclear whether the difficult work-up caused the low yield or if the reaction was simply inherently low yielding.

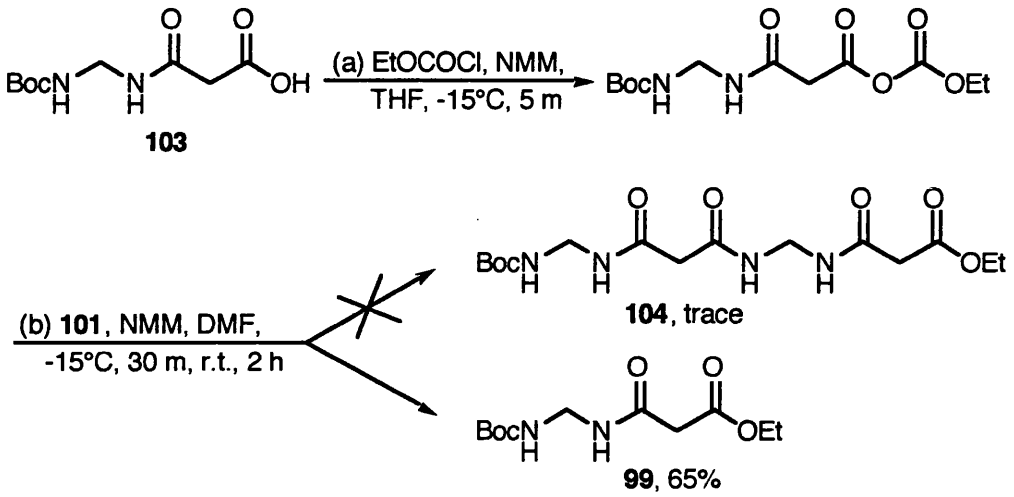
So all the available carbodiimide reagent mediated couplings proved unsatisfactory, producing the target PMRI tetrapeptide, **104**, in low yield after difficult isolation and purification procedures.

These experiments did, however, specify criteria for a more successful coupling procedure: due to the solubility properties of the target, **104**, any procedure using a partition between an organic and aqueous phase during isolation is unsuitable. A procedure with few by-products would greatly ease product isolation. Although many peptide coupling procedures have been developed, few meet these criteria.[100,242] An ideal coupling procedure would permit isolation of the target, **104**, simply by washing out impurities from the crude product precipitated during the reaction. These considerations, compounded by the generation of HOBt by many of the coupling reagents (*e.g.* BOP and HBTU),[100] weighed against further investigation of "direct" coupling reagents.

(ii) Mixed anhydride coupling

The mixed anhydride coupling procedure fulfils the above criteria, for the only by-products are an acid (or carbon dioxide and an alcohol in the case of carbonic anhydrides) and the salt of the base employed.[100] However, echoing Goodman and co-workers' earlier

result [scheme 6, section **1.5.1***(a)(i)(1)*], an attempted ethyl carbonate mixed anhydride coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Glyψ(NHCO)Gly-OEt, **101**, under standard conditions,[251] yielded only Boc-Glyψ(NHCO)Gly-OEt, **99** (65%), not the desired product, **104**: scheme 46.



Scheme 46: An attempted mixed anhydride synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**.

This result merely served to confirm that carbonate mixed anhydride procedures are unsuitable for coupling malonyl residues. None of the other mixed anhydride procedures[100] were employed in the absence of an explanation for this failure.‡

(iii) Formation and coupling of active esters

The coupling reactions employing carbodiimide reagents and auxiliary nucleophiles described above [section **2.1.1***(d)(i)*] suggested that active esters of PMRI dipeptides, formed *in situ*, are useful acylating species. Thus, the formation and isolation of such an active ester, followed by coupling, constitutes a suitable procedure for the preparation of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, since by-products may be removed in both the formation and coupling steps.

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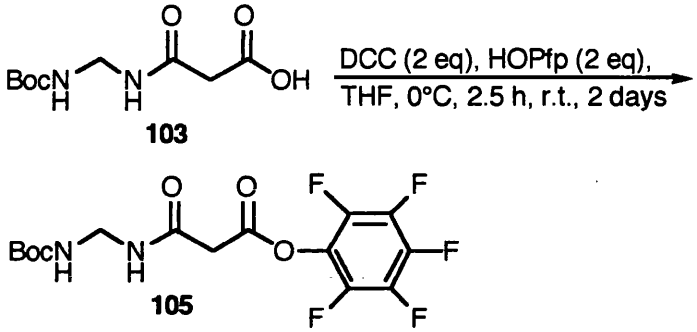
‡ For a discussion of the mechanism of this reaction see section **2.1.1***(d)(iii)(3)*. A symmetrical anhydride procedure is unfeasible because malonic acids (with a C^2 hydrogen) generally form ketene type, rather than conventional dimeric anhydrides, *e.g.* the dehydration of malonic acid itself yields carbon suboxide.[252]

(1) Pentafluorophenol

The pentafluorophenyl active ester Boc-Glyψ(NHCO)Gly-OPfp, **105**, was synthesised using the standard method and readily isolated, largely free from DCU, in 64% crude yield: scheme 47.[242]



103

105

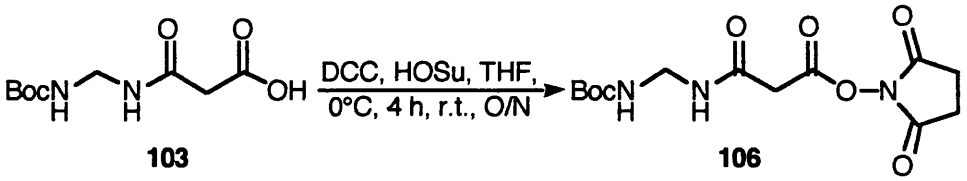
Scheme 47: DCC mediated synthesis of Boc-Glyψ(NHCO)Gly-OPfp, **105**.

However, the isolated active ester, **105**, seemed to be impure, the purity being very difficult to assess as the pentafluorophenyl moiety gives rise to no detectable signals in ^{1}H or ^{13}C NMR spectroscopy. The material appeared to be a mixture of the starting material, **103**, and the desired active ester, **105** (ratio undetermined), and TLC suggested that the active ester decomposed, further confusing the issue.

Given these difficulties, pentfluorophenol was rejected in favour of other leaving groups.

(2) HOSu / DCC

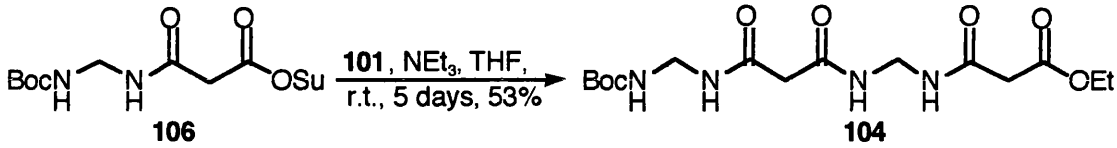
Although frequently utilised *in situ*, HOBt esters are seldom isolated, whereas HOSu esters are widely used both *in situ* and as isolated intermediates.[100] HOSu esters have been successfully employed in PMRI peptide synthesis.[124,174,253] Therefore the active ester Boc-Glyψ(NHCO)Gly-OSu, **106**, was prepared using DCC, following a standard procedure:[242] scheme 48.



103 **106**

Scheme 48: DCC mediated synthesis of Boc-Glyψ(NHCO)Gly-OSu, **106**.

The resultant active ester, **106**, (obtained in quantitative crude yield) was reasonably pure, contaminated only by traces of DCU and unreacted acid, **103** (detected by ^{1}H NMR spectroscopy and m.s.). Purification by crystallisation was possible but gave a low recovery so the active ester, **106**, was employed directly in subsequent coupling reactions. Coupling of Boc-Glyψ(NHCO)Gly-OSu, **106**, and HCl.Glyψ(NHCO)Gly-OEt, **101**, under standard conditions,[242] produced Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, in 53% yield [over the two steps from Boc-Glyψ(NHCO)Gly, **103**]: scheme 49.



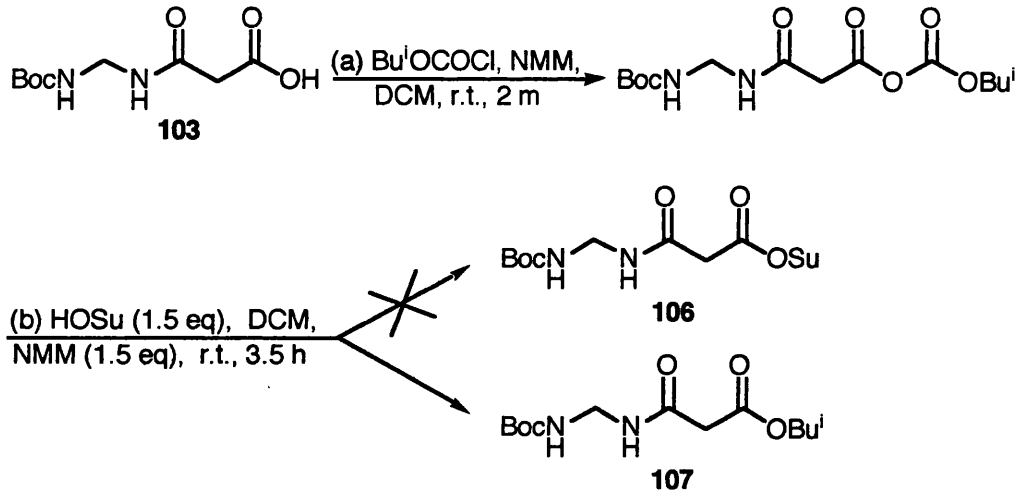
Scheme 49: Acylation of HCl.Glyψ(NHCO)Gly-OEt, **101**, with Boc-Glyψ(NHCO)Gly-OSu, **106**.

Isolation and purification of the product, **104**, were achieved, as anticipated, by washing the crude precipitate with water, followed by column chromatography.

No improvements on this coupling procedure were found; changing the reaction solvent to DMF merely complicated product isolation, resulting in a reduced yield.

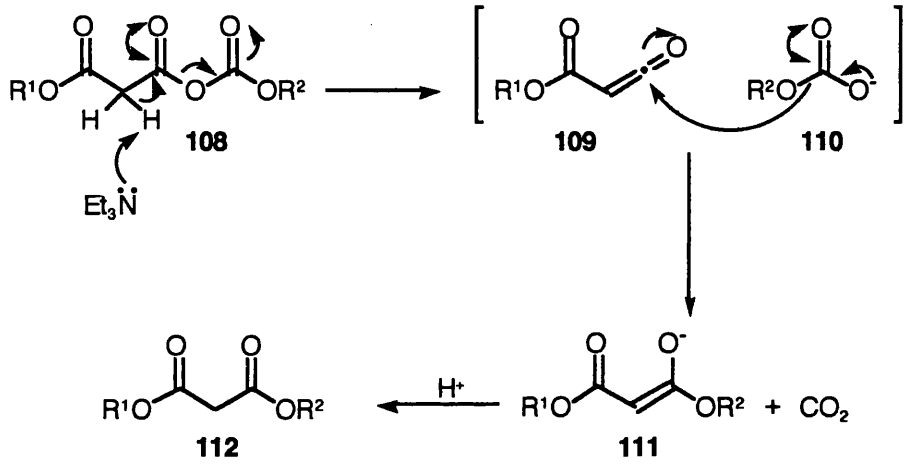
(3) Alternative synthesis of Boc-Glyψ(NHCO)Gly-OSu, 106

An alternative preparation of active esters, developed by Benoiton *et al.* uses a mixed anhydride as intermediate, rather than employing DCC.[254] However this procedure failed to give Boc-Glyψ(NHCO)Gly-OSu, **106**; the only isolated product was Boc-Glyψ(NHCO)Gly-OBu^i, **107** (in 57% crude yield): scheme 50.



Scheme 50: An attempted mixed anhydride synthesis of Boc-Glyψ(NHCO)Gly-OSu, **106**.

This failure provides further evidence, if any was needed, that carbonate mixed anhydride procedures are unsuitable for coupling malonyl residues. Indeed, the sole application of such procedures is the synthesis of malonate esters. A literature search revealed just such an application.[255,§] In the reaction, after generation of the mixed anhydride, **108**, deprotonation by base results in elimination of the monoalkyl carbonate, **110**, and the formation of a ketene, **109**. These two products then interact and the alkoxide residue of **110** is transferred to the ketene, **109**, affording the enolate, **111**, and carbon dioxide. Finally protonation yields the dialkyl malonate, **112**: scheme 51.[255]



Scheme 51: The mechanism of ester formation from malonate / carbonate mixed anhydrides, **108**.[255]

This process affords little scope for trapping the mixed anhydride with other nucleophiles (such as an amine or HOSu), so no further investigation of mixed anhydride couplings of malonyl residues was undertaken.

In summary, a selection of coupling procedures successfully yielded the desired PMRI tetrapeptide, **104** [the simplest (blocked) representative of the target compound class], the best of these being the HOSu active ester procedure described in section **2.1.1***(d)(iii)(2)*, which gave a 53% yield.

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§ The same overall reaction of a carboxylic acid and a chloroformate to yield the carboxylate ester is fairly general but requires the agency of DMAP.[256]

This yield is, however, rather moderate, though within the range of those achieved by Martinez and co-workers in their acylations using H_2N-*m*Phe-OSu [51-79%, over both an amino terminal deprotection (or *gem*-diaminoalkyl residue synthesis) and coupling step],[124,174] and close to that of Pallai *et al.* in their coupling of MeO-*m*Phe-OSu and Thr(Bu^t) (60%), from which they also recovered free acid, MeO-*m*Phe.[253] What causes this moderate yield [and the general low yields experienced in Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, synthesis] is not obvious. Many side reactions of the acid component, **103**, in the presence of carbodiimide reagents may be suspected: not only the classic side reactions such as *N*-acylurea formation (which should, however, be suppressed by the auxiliary nucleophile),[100] but also side reactions involving ketene formation [as encountered in attempted mixed anhydride procedures, see sections **1.5.1***(a)(i)(1)*, **2.1.1***(d)(ii)* and *(iii)(3)*],¶ although such a species could itself act as an acylating agent.[258]

However, the successful synthesis of the active ester, **106**, implies that the problems are not attributable to DCC. Ketene formation from **106**, in the presence of triethylamine during the coupling step is a possibility and it is not obvious where such a side reaction would lead.

Decomposition of the amino component, **101**, during (or prior to) the coupling reactions is likely. According to Loudon and co-workers' study [section **1.5.1***(a)(i)(5)*] the (ultimate) products of this decomposition are ammonia, ethyl malonamate, **118**, and formaldehyde [as discussed in section **2.1.1***(b)(i)*]. Therefore a possible by-product of the coupling reactions is Boc-Glyψ(NHCO)Gly-NH_2, resulting from ammonolysis of the acylating species (ammonia being a better nucleophile than a *gem*-diaminoalkyl residue). However, neither Boc-Glyψ(NHCO)Gly-NH_2 nor ethyl malonamate, **118**, were isolated from any of the coupling reactions.*

¶ The action of DCC / triethylamine on suitable carboxylic acids yields ketenes.[257]

* Indeed, no by-products whatsoever were isolated, though TLC indicated their presence and identified **103** (which may merely be that carried through) in the HOSu active ester procedure.

Other than the studies of the mixed anhydride procedure [see sections **1.5.1***(a)(i)(1)* and **2.1.1***(iii)(3)*], no investigation of side reactions during PMRI peptide coupling reactions has been undertaken. Nonetheless it seems reasonable to conclude that the moderate yields of these reactions are caused by *gem*-diaminoalkyl residue decomposition and difficult isolation and purification of the product, **104**, due to its poor solubility properties.

The low solubility of **104** necessitated NMR studies at low concentration (*ca.* 13 mM) in $DMSO_{d6}$. These conditions make conformational studies, the second aspect of this project, difficult. Therefore two other PMRI tetrapeptides [rather than Boc-Alaψ(NHCO)Gly-Alaψ(NHCO)Gly-OEt] with lipophilic side chains, were targeted, in order to produce members of the target compound class with higher solubility in organic solvents.

2.1.2 *Synthesis of Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, 122.*

Valine, with its *iso*-propyl side chain, is more lipophilic than glycine or alanine. In addition its side chain is β-branched, a property which contributes to β-sheet stabilisation.[13,187,204,259,260] Therefore valine replaced alanine, the original second choice, as a side chain bearing *gem*-diamino residue.† Thus, the PMRI tetrapeptide Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, was regarded as an appropriate target because it was expected to be more soluble in organic solvents than **104** and the side chains are in specified positions (*i.e.* on every other residue and not on the configurationally labile malonyl residues) appropriate to stabilise β-sheet formation (in this or subsequent, elongated PMRI peptides),[204] a property of interest in this project.

The same approach to Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, as to Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, was used, *i.e.* the coupling of two protected Valψ(NHCO)Gly moieties.

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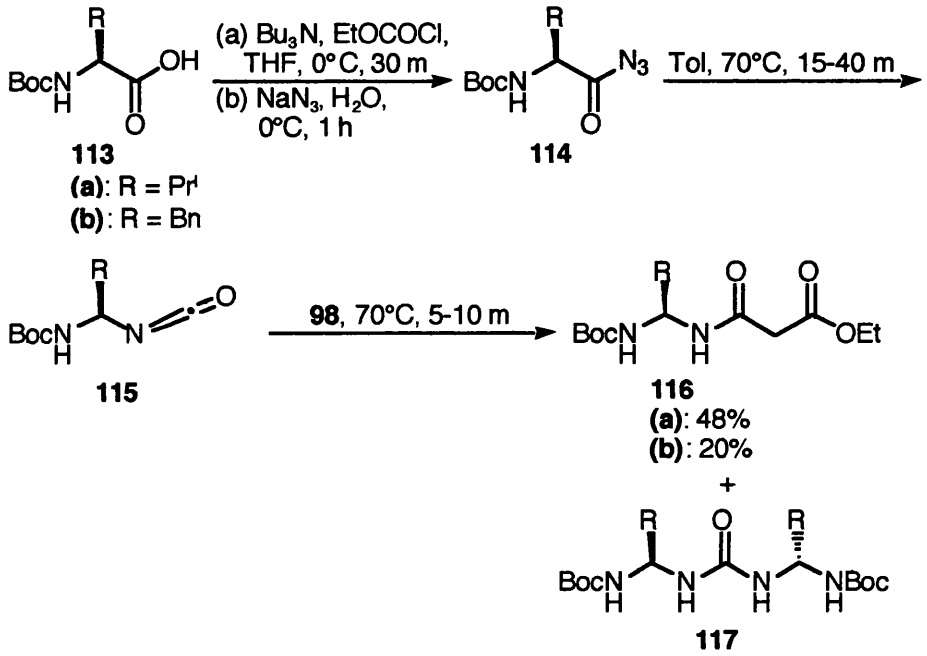
† Alanine has a conformational preference for α-helices rather than β-sheets.[13,187,204,259,260]

(a) Synthesis of Boc-Valψ(NHCO)Gly-OEt, 116(a)

(i) The Goldschmidt and Wick type procedure

The Goldschmidt and Wick type procedure provided Boc-Valψ(NHCO)Gly-OEt, **116(a)**, in 48% yield from Boc-valine, **113(a)**: scheme 52.



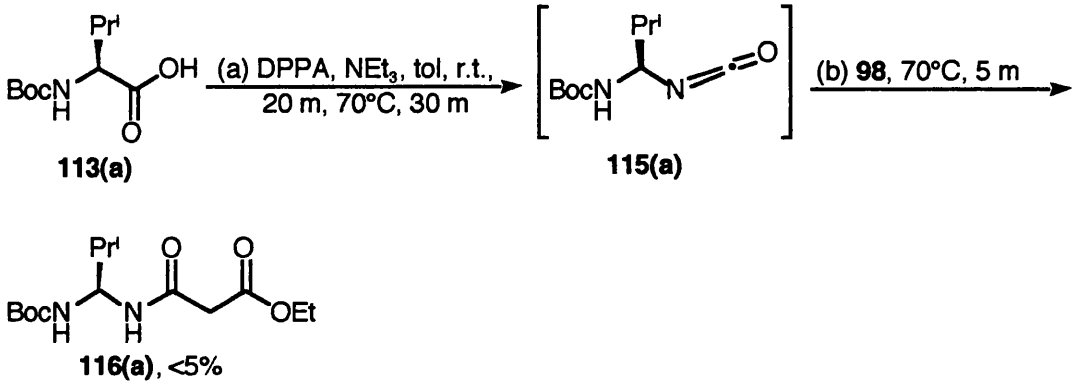
Scheme 52: Goldschmidt and Wick type synthesis of Boc-Valψ(NHCO)Gly-OEt, **116(a)** and Boc-Pheψ(NHCO)Gly-OEt, **116(b)**.

Analogous to the Boc-Glyψ(NHCO)Gly-OEt, **99**, case, Boc-valine, **113(a)**, monoethyl malonate, **98**, and the urea, **117(a)**, were identified as by-products (by TLC or ^{1}H NMR spectroscopy), although purification of Boc-Valψ(NHCO)Gly-OEt, **116(a)** was less difficult. In order to depress regeneration of Boc-valine, **113(a)**, by hydrolysis during aqueous sodium azide addition, alternative solvents were employed. However, both addition of sodium azide suspended in DMF or dissolved in acetone / water resulted in decreased yields of Boc-Valψ(NHCO)Gly-OEt, **116(a)** (29% and 45% respectively).

(ii) DPPA

As described in section **1.5.1***(a)(i)(1)*, DPPA may be used in the synthesis of *gem*-diaminoalkyl residues. A Goldschmidt and Wick type synthesis of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, employing DPPA (based upon the procedures of Goodman and Chorev[90] and Verdini and co-workers[112]) using IR spectroscopy to monitor the consumption of DPPA and accumulation of isocyanate **115** prior to addition of

monoethyl malonate, **98**, (analogous to our normal procedure, see experimental chapter) failed. The IR spectroscopy did indicate consumption of DPPA, some accumulation of isocyanate **115(a)**, and its subsequent disappearance after monoethyl malonate, **98**, addition, but little product, **116(a)**, was isolated (<5% crude yield): scheme 53.



113(a) **115(a)** **116(a)**, <5%

Scheme 53: Attempted synthesis of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, using DPPA.

It is noteworthy that DPPA is more frequently used to generate isocyanates (or acyl azides for that matter) *in situ* rather than in isolation.[110,111,261] Presumably a slow decomposition of these species occurs in the presence of DPPA (or its by-products) and the absence of a trapping nucleophile. No identifiable products were isolated from these reactions to enable elucidation of the reaction pathway.

Thus no improvements on the usual Goldschmidt and Wick type synthesis of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, were made.

(b) N-*Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)***

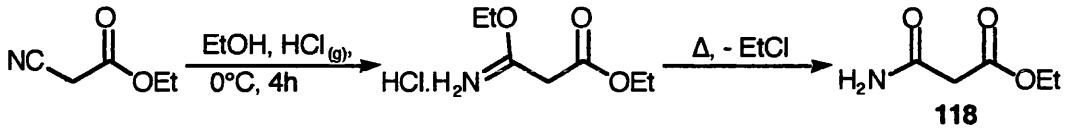
(i) Deprotection using hydrogen chloride

Treatment of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, with hydrogen chloride in ethanol or 1,4-dioxane effected Boc removal and partial decomposition. Attempts to obtain pure HCl.Valψ(NHCO)Gly-OEt from the resultant mixture of products {by ether trituration, ethyl acetate extraction of an aqueous solution, ion-exchange chromatography [Amberlite IR 120(+)], crystallisation or HPLC} failed. However, column chromatography did provide a sample of decomposition product. This isolated material turned out to be a mixture of ethyl malonamate, **118**, and ethyl malonyl-Valψ(NHCO)Gly-OEt [bis-(*N*-ethylmalonyl)-1,1-diamino-2-methylpropane], **120**, as indicated by ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy and m.s. The sample of

mixed by-products was insufficient to permit separation, therefore their independent synthesis was undertaken, in order to confirm their identity.

*(1) Synthesis of ethyl malonamate, **118***

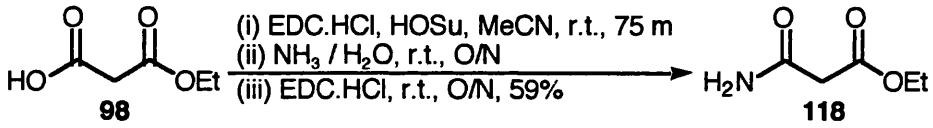
The literature synthesis of ethyl malonamate, **118**, is that due to Pinner, from ethyl cyanoacetate: scheme 54.



118

Scheme 54: Pinner's synthesis of ethyl malonamate, **118**.[262]

However, having monoethyl malonate, **98**, in hand, a simple EDC / HOSu mediated coupling with aq. ammonia was carried out, which provided ethyl malonamate, **118**, in 59% yield: scheme 55.

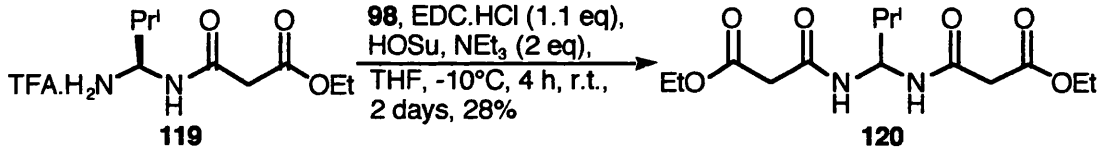


98 **118**

Scheme 55: Coupling of monoethyl malonate, **98**, and ammonia.

*(2) Synthesis of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120***

In addition to the desire to confirm the above by-products' identity, ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, was deemed inherently interesting as a truncated member of the target compound class, as illustrated by the formula EtO-*m*Gly-*g*Val-*m*Gly-OEt. EDC / HOSu mediated coupling of monoethyl malonate, **98**, and TFA.Valψ(NHCO)Gly-OEt, **119**, provided ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, in 28% yield: scheme 56.



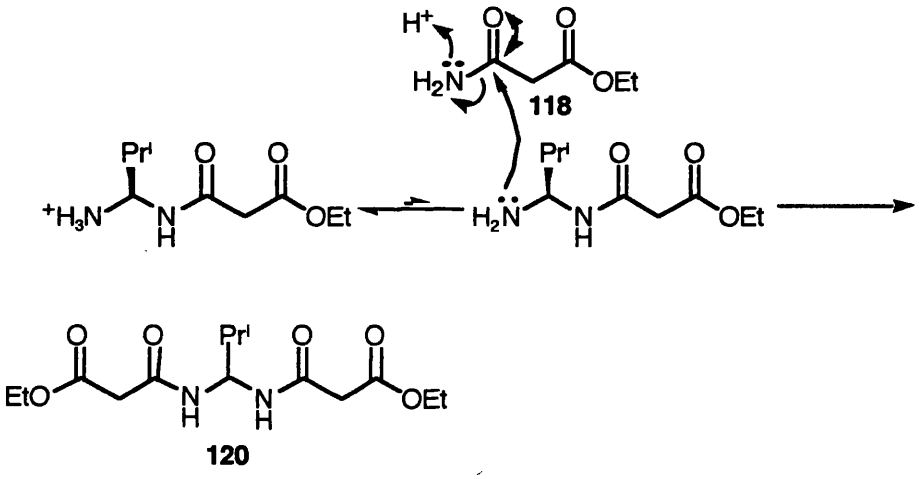
119 **120**

Scheme 56: Coupling of monoethyl malonate, **98**, and TFA.Valψ(NHCO)Gly-OEt, **119**.

The low yield resulted due to difficult isolation of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, from ethyl malonamate, **118** (either carried through with, or formed by *in situ* decomposition of TFA.Valψ(NHCO)Gly-OEt, **119**). Use of DCC instead of EDC gave a lower yield (18% crude).

^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, TLC and m.s. confirmed that ethyl malonamate, **118**, and ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, were indeed the by-products isolated during HCl.Valψ(NHCO)Gly-OEt formation.

Thus we may conclude that *N*-terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, with hydrogen chloride solutions results in some decomposition to yield a mixture of the desired compound, plus ethyl malonamate, **118**, and ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**. The ethyl malonamate, **118**, presumably derives from HCl.Valψ(NHCO)Gly-OEt, according to the mechanism determined by Loudon and co-workers {scheme 19, section **1.5.1***(a)(i)(5)* [for HCl.Valψ(NHCO)Gly-OEt: R^1 = EtO_2CCH_2, R^2 = Pr^i and R^3 = H]‡}.[116] However, this does not account for the formation of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, which most probably arises from a transamidation reaction between HCl.Valψ(NHCO)Gly-OEt and ethyl malonamate, **118** [or a second molecule of HCl.Valψ(NHCO)Gly-OEt], under the acidic conditions: scheme 57.



Scheme 57: Transamidation reaction leading to ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**.

(ii) Deprotection using TFA

N-Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, using TFA (with or without TES) furnished TFA.Valψ(NHCO)Gly-OEt, **119**, in 84% crude yield (in all cases): scheme 58.

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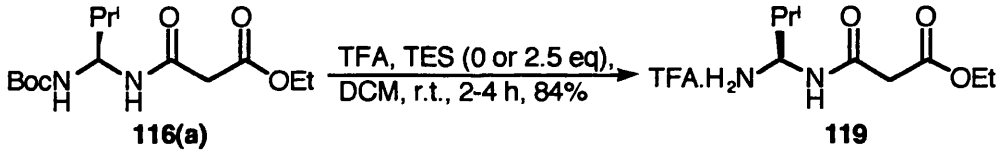
‡ Note that the stereochemistry of HCl.Valψ(NHCO)Gly-OEt is opposite to that depicted in scheme 19.



Scheme 58: *N*-Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, with TFA (/ TES).

The preferred method required no TES and utilised an alternative work-up procedure employing petrol to aid TFA removal and therefore prevent excessive dissolution of TFA.Valψ(NHCO)Gly-OEt, **119**, during ether trituration (see experimental chapter).[263] Some decomposition occurred under these conditions (as indicated by TLC), but the resultant product was sufficiently pure for use in the subsequent coupling step (as shown by ^{1}H NMR spectroscopy).

Thus, as in the Boc-Glyψ(NHCO)Gly-OEt, **99**, case, TFA is superior to hydrogen chloride solutions for *N*-Boc deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**. Presumably decomposition is a greater problem during the *N*-deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, than of Boc-Glyψ(NHCO)Gly-OEt, **99**, due to the *iso*-propyl group in **116(a)** stabilising the resultant iminium species [see section **1.5.1***(a)(i)(5)*].

*(c) C-Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)***

Saponification of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, gave the free acid, Boc-Valψ(NHCO)Gly, **121**, in 99% yield.

*(d) Coupling of Boc-Valψ(NHCO)Gly, **121**, and TFA.Valψ(NHCO)Gly-OEt, **119***

(i) Carbodiimide reagent mediated coupling

(1) DCC / HOBt

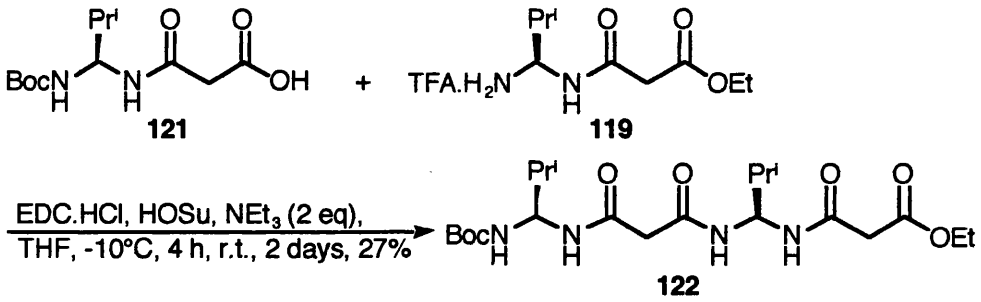
Unlike Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, was expected to be sufficiently soluble in organic solvents to permit its efficient extraction during the standard work-up of a DCC / HOSu mediated coupling procedure.

Thus Boc-Valψ(NHCO)Gly, **121**, and TFA.Valψ(NHCO)Gly-OEt, **119**, were coupled under the usual conditions (*i.e.* as scheme 44). However, little crude Boc-

Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, was isolated on standard work-up,[242] indicating that it is not very soluble in organic solvents after all.

(2) EDC / HOSu

Given the disappointingly poor solubility of Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, the methods developed for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, were applied. So Boc-Valψ(NHCO)Gly, **121**, and TFA.Valψ(NHCO)Gly-OEt, **119**, were coupled using EDC / HOSu, scheme 59, and the product recovered by evaporation of solvent, washing the residue with water and chromatography of the resultant precipitate to yield Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122** (27%).



Scheme 59: EDC / HOSu mediated coupling of Boc-Valψ(NHCO)Gly, **121**, and TFA.Valψ(NHCO)Gly-OEt, **119**.

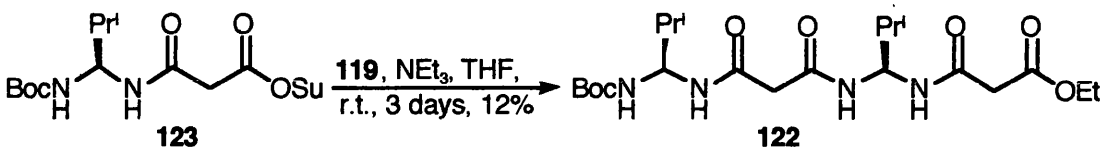
Although better than the corresponding synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, this yield was poor, presumably for similar reasons, *i.e.* TFA.Valψ(NHCO)Gly-OEt, **119**, decomposition, difficult product isolation and possibly other inherent complications [*c.f.* sections **2.1.1***(d)(i)(3)* and *(d)(iii)(3)*]. Again, no indicative by-products were identified, other than Boc-Valψ(NHCO)Gly, **121**.

(ii) Formation and coupling of the HOSu active ester, Boc-Valψ(NHCO)Gly-OSu, 123

The HOSu active ester procedure was the method of choice for the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, therefore this procedure was applied to the synthesis of Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**.

(1) HOSu / DCC

Boc-Valψ(NHCO)Gly-OSu, **123**, was prepared in 87% crude yield using essentially the same procedure as for Boc-Glyψ(NHCO)Gly-OSu, **106** [section **2.1.1***(d)(iii)(2)*]. The resultant active ester, **123**, contaminated by traces of DCU and HOSu (as indicated by ^{1}H NMR spectroscopy), was employed without purification in a coupling reaction with TFA.Valψ(NHCO)Gly-OEt, **119**, under the usual conditions: scheme 60.



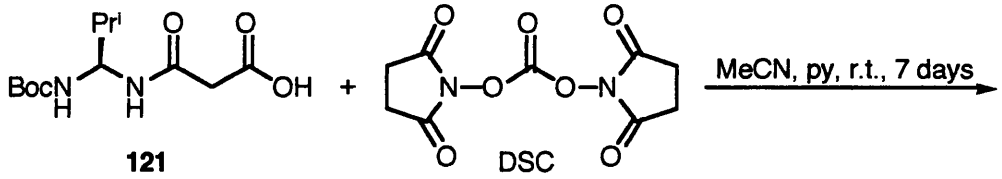
123 **122**

Scheme 60: Acylation of TFA.Valψ(NHCO)Gly-OEt, **119**, with Boc-Valψ(NHCO)Gly-OSu, **123**.

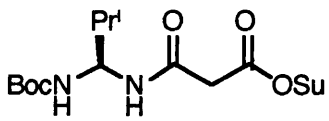
Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, was thus obtained in only 12% yield [over the two steps from Boc-Valψ(NHCO)Gly, **121**].

(2) DSC

In an attempt to increase this low yield, a different synthesis of Boc-Valψ(NHCO)Gly-OSu, **123**, was undertaken. Ogura and co-workers have developed a number of reagents which convert *N*-protected amino acids and peptides to HOSu active esters [*i.e.* *N,N'*-disuccinimidyl carbonate (DSC),[264] *N*-succinimidyl diphenylphosphate,[265] and *N,N'*-disuccinimidyl oxalate[266]]. Thus, the reaction of DSC (3 eq, added in 3 batches during the course of the reaction) and Boc-Valψ(NHCO)Gly, **121**, provided Boc-Valψ(NHCO)Gly-OSu, **123**, in quantitative crude yield: scheme 61.



121



123

Scheme 61: Synthesis of Boc-Valψ(NHCO)Gly-OSu, **123**, using DSC.

TLC and ^{1}H NMR spectroscopy indicated that the resultant active ester, **123**, was of higher purity than that obtained using DCC, contaminated only by traces of impurities (probably including the free acid, **121**). The subsequent coupling reaction [under essentially the same conditions as before (scheme 60)] of the active ester, **123** (used without purification), with TFA.Valψ(NHCO)Gly-OEt, **119**, provided a reasonably pure sample of Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, in 27% yield, without the need for chromatography [**122** was isolated by evaporating the reaction mixture to dryness, washing the resultant off-white solid with water and drying under high vacuum over phosphorus pentoxide (*i.e.* the usual work-up)]. This procedure was not thoroughly investigated but holds promise for future development.

In summary, Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, may be synthesised via the active ester, **123** (generated *in situ* with EDC / HOSu or in isolation with DSC) in moderate yield. Presumably difficulties similar to those encountered with the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** [section **2.1.1***(d)*], augmented by problems associated with the *iso*-propyl group [*i.e.* additional steric hindrance and greater decomposition of TFA.Valψ(NHCO)Gly-OEt, **119**], account for the low yield.

2.1.3 *Synthesis of Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, 126.*§

Phenylalanine is highly lipophilic, so the synthesis of Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, was undertaken (following the established route) with the expectation that it would be more soluble in organic solvents than the PMRI tetrapeptides **104** and **122**, described above. Furthermore, phenylalanine has a high β-sheet forming tendency.[13,187,204,259]

(a) Synthesis of Boc-Pheψ(NHCO)Gly-OEt, 116(b)

The Goldschmidt and Wick type procedure provided Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, in 20% yield from Boc-phenylalanine, **113(b)**: scheme 52. Again Boc-

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§ The work described in this section was performed in conjunction with three final year undergraduate project students.[267-269]

phenylalanine, **113(b)**, monoethyl malonate, **98**, and the urea, **117(b)**, were identified as by-products (by TLC or ^{1}H NMR spectroscopy). An additional by-product, *N*-(Boc)-1-amino-*trans*-styrene [*N*-(Boc)-2-phenyl-*trans*-ethenamine], **24(b)**, was isolated in this case, presumably formed by the mechanism proposed by Chorev, Goodman and MacDonald [scheme 5, section **1.5.1***(a)(i)(1)*].[105] No significant procedural changes were attempted [as none of those tested on the synthesis of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, proved worthwhile: see section **2.1.2***(a)*] and therefore no improvement on this low yield was attained. The unusually low yield in this case is attributed, at least in part, to the ease with which the Curtius rearrangement of acyl azide **114(b)** occurred (during its isolation effervescence was observed on standing at r.t.) thus increasing the opportunity for side reactions prior to product, **116(b)**, formation.

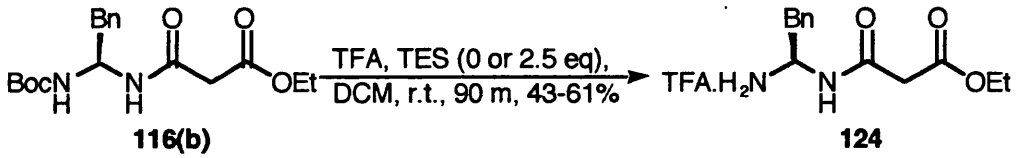
(b) N-*Terminal deprotection of Boc-Pheψ(NHCO)Gly-OEt, 116(b)*

(i) Deprotection using hydrogen chloride

In view of the by-product formation experienced during *N*-Boc deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, and Boc-Valψ(NHCO)Gly-OEt, **116(a)**, using ethanolic hydrogen chloride [section **2.1.1***(b)(i)* and **2.1.2***(b)(i)*] it was not surprising when the same procedure caused substantial decomposition of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**: no products were isolated.

(ii) Deprotection using TFA

N-Boc deprotection of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, using TFA (with or without TES) furnished TFA.Pheψ(NHCO)Gly-OEt, **124**: scheme 62.



116(b) **124**

Scheme 62: *N*-Terminal deprotection of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, with TFA (/ TES).

The highest yield obtained was in the presence of TES (61% vs 43% in its absence). In both cases significant product isolation difficulties occurred due to dissolution of the

product, **124**, in ether in the presence of residual TFA. However, in the absence of TES a pure sample of Boc-Pheψ(NHCO)Gly-OEt, **124**, resulted after crystallisation.

Thus TFA again proved superior to hydrogen chloride solutions for *N*-Boc deprotection. However the yields were rather unsatisfactory in this case: presumably the benzyl side chain provides additional stabilisation for the iminium species formed during decomposition [see scheme 19, section **1.5.1***(a)(i)(5)*], though no by-products were isolated to support this supposition.

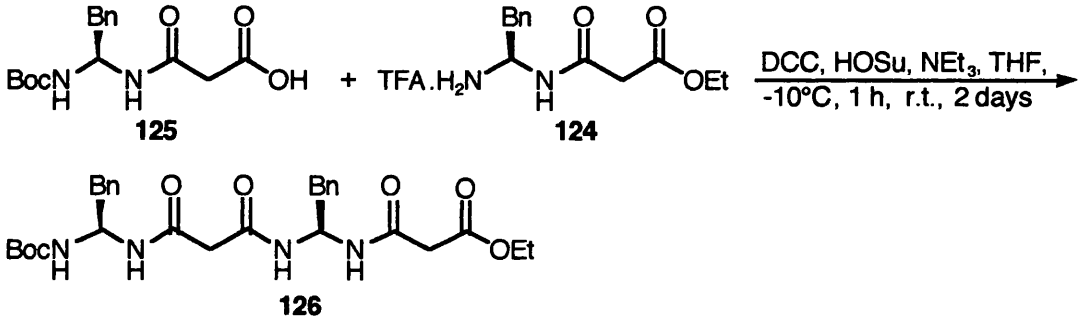
*(c) C-Terminal deprotection of Boc-Pheψ(NHCO)Gly-OEt, **116(b)***

Saponification of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, gave the free acid, Boc-Pheψ(NHCO)Gly, **124**, in 88% yield.

*(d) Coupling of Boc-Pheψ(NHCO)Gly, **125**, and TFA.Pheψ(NHCO)Gly-OEt, **124***

(i) DCC / HOSu

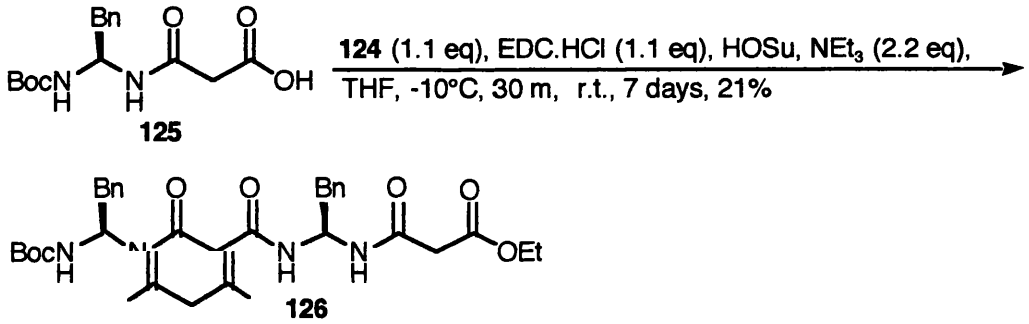
Boc-Pheψ(NHCO)Gly, **125**, and TFA.Pheψ(NHCO)Gly-OEt, **124**, were coupled following the standard procedure to provide Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, in 55% crude yield (contaminated by DCU), scheme 63; in this case the product was isolated by extraction (with ethyl acetate).



Scheme 63: DCC / HOSu mediated coupling of Boc-Pheψ(NHCO)Gly, **125**, and TFA.Pheψ(NHCO)Gly-OEt, **124**.

(ii) EDC / HOSu

In order to procure the target PMRI tetrapeptide, **126**, free from DCU, the coupling reagent was changed to EDC, scheme 64, which furnished pure Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, in 21% yield, after isolation by extraction and chromatography.



Scheme 64: EDC / HOSu mediated coupling of Boc-Pheψ(NHCO)Gly, **125**, and TFA.Pheψ(NHCO)Gly-OEt, **124**.

Thus Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, was synthesised in poor yield, again attributed to decomposition of TFA.Pheψ(NHCO)Gly-OEt, **124**. In both coupling reactions the only by-product identified was the free acid, **125**. Although it was possible to isolate Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, by extraction, it nevertheless displayed low solubility in organic solvents and NMR experiments still had to be performed in $DMSO_{d6}$.

Due to the particularly low yields suffered in the synthesis of Boc-Pheψ(NHCO)Gly-OEt, **116(b)** and its *N*-Boc deprotection, no further work was carried out on this Phe series.

2.1.4 *Solubilisation.*

In order to aid chain extension of the PMRI tetrapeptides **104** and **122** in solution, an investigation of possible solubilisation strategies was undertaken.

(a) Solvents and additives

(i) Polar solvents

Toniolo and co-workers established by extensive studies (using quantitative IR absorption titrations) that the major cause of insolubility of blocked peptides is intermolecular self-association, often with formation of a regular β-structure.[270] Their results show that increasing peptide chain length (from dipeptide through to hexapeptide) leads to increasing self-association, whereas further extension (hexapeptide through to decapeptide) reverses this trend.[270] If these results obtained with natural peptides (*C*-terminal segments of porcine secretin)[270] apply to PMRI peptides then they imply that

some solubilisation strategy is essential, for the solubility of elongated PMRI peptides is expected to decline before it increases. This is a reasonable assumption because solubility in organic solvents decreases markedly through the PMRI dipeptide to tetrapeptide series: **116(a)** > **120** > **122**; however further extension may not eventually lead to increasing solubility because this phenomenon for natural peptides is thought to result from a β-sheet to helix conformational transition.[271,272] This is unlikely to occur with our target structural motif [see section **1.5.3***(b)(ii)* and *(iii)*]. Toniolo and co-workers assessed the β-structure disruption potential displayed by numerous polar solvents.[270,273] They concluded that DMSO destabilises self-association more effectively than DMF or hexamethyl phosphoramide (HMPA).[270] The fluorinated alcohols 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) also strongly disrupt self-association, but a direct relationship between peptide solubility and coupling reaction yields does not exist.[273]

Narita and co-workers performed comprehensive investigations (also using quantitative IR absorption titrations plus solubility classification) of the β-structure stability of protected peptides.[271,274-277] They classified organic solvents based upon their solvating potential for protected peptides and related this classification to the solvents' electron-acceptor and -donor numbers (AN and DN).[275,278] In general, increasing the solvent AN or DN increases the solvating potential.[275] Narita and co-workers divided the solvents they investigated into six groups.[275] The fifth group [py, DMF, *N*-methyl-2-pyrrolidinone (NMP), DMSO and tributylphosphine oxide] displayed high solvating potential for blocked peptides up to heptapeptides, but poorer solvating potential for longer peptides.[275] The sixth group (acetic acid, TFE, phenol, HFIP) displayed high solvating potential even for blocked peptides longer than an octapeptide.[275]

Qualitative tests on the solubility of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, in a range of solvents were performed. In ethyl acetate, ethanol, water, THF and dichloromethane (members of Narita and co-workers' second, third and forth groups)[275] it is sparingly soluble. Whereas it is fairly soluble in DMF and DMSO and soluble in TFE and HFIP. Therefore elongation reactions may be performed in DMF,

which is preferred over DMSO due to greater ease of removal (although DMSO may be necessary for further elongations as it reduces self-association better than DMF).[270] DMF is favoured over TFE and HFIP. The latter has been described as unsuitable for carbodiimide mediated couplings,[279] which suggests that TFE may also be unsuitable. Both these fluoroalcohols can form their respective esters (which are weak acylating agents) during coupling reactions.[277,280,¶] However, given that TFE or HFIP and dichloromethane mixtures are reportedly as, or more effective than the pure fluoroalcohols for dissolving peptides of low solubility,[274,277] a result affirmed by the dissolution of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, in a mixture of HFIP and dichloromethane, such mixtures may be useful for elongation reactions if DMF or DMSO prove unsuccessful.

(ii) Additives: chaotropic salts and lithium salts

Chaotropic salts are substances that destroy the order of water when dissolved in it and thereby raise the solubility of hydrophobic substances in the solution. They have proved useful in tackling the problem of "difficult sequences" in SPS (*e.g.* potassium thiocyanate or sodium perchlorate in DMF / dichloromethane).[283]

Seebach and co-workers discovered that lithium halides and peptides co-solubilise each other in THF.[288,*] The effect is reversed (*i.e.* precipitation occurs) upon addition of water.[288] The effect is caused by an interaction between lithium ions and (amide)

¶ TFE and more so HFIP have proved useful as resin swelling solvents during SPS of so-called "difficult sequences",[279,281] which are the SPS manifestation of solution phase insolubility. "Difficult sequences" are caused by incomplete solvation and thus self-association of resin bound peptides, again often with formation of β-structure.[274,282-284] However carbodiimide mediated coupling reactions are rarely carried out in the presence of fluoroalcohols, except under extreme circumstances.[273,285,286] These fluoroalcohols have been successfully used as (components of) chromatographic eluents during the purification of peptides of low solubility.[285,287]

* These authors investigated various inorganic salts, of which lithium chloride, lithium bromide, lithium iodide, lithium perchlorate, lithium tetrafluoroborate, titanium(IV) ethoxide and titanium(IV) *iso*-propoxide were most effective.[288] The effect is also observed, to a greater or lesser extent, in other solvents: dioxane, dimethoxyethane, polyethylene glycol 200, acetonitrile, DMF, NMP, 3,4,5,6-tetrahydro-1,3-dimethylpyrimidin-2(1*H*)-one (DMPU), and water (for proteins).[288-290]

carbonyl oxygens that prevents the oxygens from participating in hydrogen bonds and lowers the barrier to rotation about the CO-N bond.[291] Seebach and co-workers further developed conditions for the exploitation of this co-solubilisation during coupling reactions in solution and SPS.[290,292] Lansbury and co-workers utilised this co-solubilisation in their work on the notoriously insoluble amyloid β-plaque peptides. They found that lithium bromide in THF was an efficient solvent in this context (whereas TFE and 6 M aq. guanidinium thiocyanate were less successful) and applied it to coupling and deprotection reactions and as a solvent in gel permeation purifications.[4,293,294]

Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, readily dissolves in 1 M lithium chloride in THF, but the addition of water causes precipitation. The ^{1}H NMR spectrum of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, in lithium chloride / THF_{d8} [figure A3(b)] differs from that taken in $DMSO_{d6}$ [figure A3(a)]. These changes are similar to those described by Seebach and co-workers for a normal peptide, *i.e.* the NH signals are shifted downfield.[288] Thus, the interactions between Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, and lithium chloride are similar to those between the salt and normal peptides. Hence lithium chloride / THF provides another possible choice of solvent for coupling reactions leading to elongation of the PMRI tetrapeptides **104** and **122**.

(b) Complexes

Complex formation by peptides is rather common, *e.g.* Schneider and Kelly synthesised a modified peptide that adopts a β-sheet structure upon coordination of copper(II).[295] 1,3-Dicarbonyl compounds also readily form complexes with transition metals. Since the results with lithium chloride (described above) demonstrated the ability of the carbonyl groups in Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, to interact with lithium ions, an investigation of complex formation between **104** and copper(II) and nickel(II) was undertaken, with the view that chelation of a metal ion by **104** could result in its disaggregation and thus solubilisation.

In our hands all attempts to form a copper(II) complex of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, failed: stirring at r.t. with copper(II) chloride or copper(II)

tetrafluoroborate in DMF (with or without sodium hydroxide) merely produced a physical mixture (indicated by an unchanged IR spectrum after removal of DMF).†

Overnight stirring at r.t. of a mixture of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, and nickel(II) chloride hexahydrate in DMF, followed by evaporation produced a blue film that gave an altered IR spectrum with respect to that of **104** itself. However, further attempts to characterise this material were inconclusive and no firm statement regarding its nature (complex or physical mixture) can be made. Addition of water to this material followed by freeze-drying caused it to separate into a mixture of white and blue precipitates: the IR spectrum of the white portion was identical to that of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**. Addition of THF to the blue film produced a mixture of white and green precipitates. Therefore it seems that even if this blue film was a complex formed between nickel(II) and Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, it would not be useful for solubilisation or extraction purposes. The investigations of complex formation ceased here.

(c) Protecting groups

The solubilising influence of terminal protecting groups on all but the shortest peptides is rather limited, even for macromolecular protecting groups.[272] Therefore no investigation of alternative terminal protecting groups was undertaken [except for SPS: see section **2.2.2**].‡

Temporary blocking of backbone amide bonds of peptides deters aggregation through hydrogen bonding. However, as Greene and Wuts state: "Protection of the amide -NH is an area of protective group chemistry that has received little attention, and as a consequence few methods exist for amide -NH protection."[296] Furthermore, most of the effort directed towards the development of amide protecting groups for use in peptides has focused on protection of the asparagine and glutamine side chains.[297] Nevertheless a

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† NB: 2,4-Pentanedione did form a copper(II) complex on stirring at r.t. with copper(II) chloride in DMF.

‡ The simplest possible *N*-terminal protecting group change, from Boc to Z, was expected to aggravate the solubility problems.[270]

number of protecting groups suitable for temporary protection of backbone amide bonds have been developed, interest in this area having been revitalised by the preponderance of "difficult sequences" in SPS. The successful, solubilising, temporary, backbone amide protecting groups are: 2-hydroxy-4-methoxybenzyl (Hmb),[298-300] 2-hydroxybenzyl (Hbz),[301] 4-methoxybenzyl (pMB),[302] 2,4-dimethoxybenzyl (Dm or Dmob),[302-307] 2,4,6-trimethoxybenzyl (Tmob),[305,307] 4-methoxy-2,3-dihydroxybenzyl (DHMB),[308] ferrocenylmethyl (Fem),[309,310] cyclohexadienyliumiron(0) tricarbonyl (Fed),[311] Boc,[312,313] Bn,[302,314-316] and Bartl *et al.*'s range of groups derived from Mannich reactions (of general structure R-X-CH_2-, *e.g.* R-X = methyloxy or phenylthio).[317] However, most of these protecting groups (*i.e.* all except Boc, Bn, and those of Bartl *et al.*) are introduced by reaction at the amino terminus followed by a peptide coupling reaction that is greatly decelerated by the steric hindrance they present. Even the Hmb group, designed to aid the approach of the acylating species, suffers from slow couplings.[298,299,301]

Therefore, given the slow couplings and connected problems due to side reactions suffered *en route* to the PMRI tetrapeptides **104**, **122** and **126**, any measure expected to further impede rapid coupling was deemed unwise.

(i) Exhaustive tert*-butoxycarbonylation of Boc-Valψ(NHCO)Gly-OEt,* ***116(a)***

Ragnarsson and co-workers' method of *tert*-butoxycarbonylation permits protection at the amide itself, rather than *via* reductive amination and subsequent amidation.[312,313] Thus, a suitable solubilisation strategy is available that does not necessitate an excessively hindered coupling step. Therefore the synthesis of Boc_2-Xaaψ[N(Boc)CO]Yaa-OEt and its subsequent carboxy terminal deprotection and extension [by coupling to Xaaψ(NHCO)Yaa-OEt, *etc.* as usual] was planned.§ This level of backbone amide protection is expected to be sufficient to prevent aggregation as previous studies indicate that blocking *ca.* 1 in 6 backbone amides is adequate.[298,300,305]

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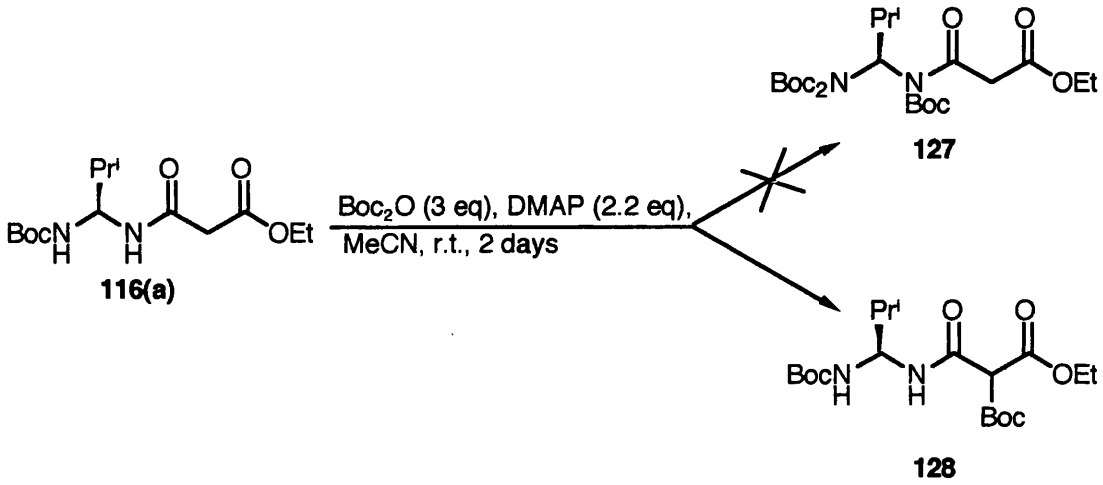
§ The Boc groups may ultimately be removed with TFA.[312] The free acid may be obtained by saponification of Boc_2-Xaaψ[N(Boc)CO]Yaa-OEt,[318] though alternative carboxy protection strategies might have proved necessary.[313]

Thus Boc-Valψ(NHCO)Gly-OEt, **116(a)**, was employed as a model substrate in an exhaustive *tert*-butoxycarbonylation reaction under Ragnarsson and co-workers' conditions,[312] *i.e.* an attempted synthesis of Boc2-Valψ[N(Boc)CO]Gly-OEt, **127**: scheme 65.



Scheme 65: Exhaustive *tert*-butoxycarbonylation of Boc-Valψ(NHCO)Gly-OEt, **116(a)**.

However, this reaction effected not the expected *N*-acylation, but rather *C*-acylation, to produce ethyl *N*-[(*S*)-1-(*N'*-Boc-amino)-2-methylpropyl]-(*R,S*)-2-Boc-malonamate, **128**, in 56% yield, plus 9% recovered starting material, **116(a)**. Whether the *C*-acylated product, **128**, might have proved useful in preventing aggregation is open to conjecture: no further investigation was performed as it gradually reverted to starting material, **116(a)**, during characterisation. No further attempts at exhaustive *tert*-butoxycarbonylation were made because the results of Ragnarsson and co-workers indicated that *C*-acylation of malonates under these conditions is general and subsequent *N*-acylation is very sluggish.[319]

This result suggested that the conditions required for benzylation (strong base / benzyl bromide),[314] and for Bartl *et al.*'s protection method (paraformaldehyde / R-X-H / Δ)[317] would also result in undesired *C*-derivatisation, so they were not investigated.

*(ii) TBDMS "protection" of Boc-Glyψ(NHCO)Gly-OEt, **99***

Instead of *N*-protection, an attempt to derivatise the malonyl residue(s) was undertaken: since the malonyl residues of PMRI peptides readily enolise [see section **1.5.1***(a)(ii)(7)*] the synthesis of a silyl ketene acetal (or *O,N*-acetal) would prevent the

carbonyl oxygen from participating in hydrogen bonding and thus prevent aggregation. Thus Boc-Glyψ(NHCO)Gly-OEt, **99**, as a model substrate, was treated with TBDMSOTf and triethylamine in dichloromethane.[320,321] It was unclear where silylation might occur (the amide oxygen seeming most probable, but the ester carbonyl oxygen or the nitrogens were possibilities);[320] nor whether *N*-Boc deprotection might occur.[322] The reaction was monitored by TLC (mixture A). After 30 m at 0°C TLC indicated a mixture of starting material, **99**, (R_F = 0.41) and a higher running product (R_F = 0.68). The reaction was allowed to warm to r.t. and after a further 30 minutes TLC showed another product spot (R_F = 0.50). No further change occurred after another hour at r.t. so the reaction was worked up following the standard procedure (*i.e.* diluted with dichloromethane, washed with cold sat. sodium bicarbonate, the organic layer dried over sodium sulfate, evaporated, the residue taken up in ether, filtered and evaporated).[320,321] Column chromatography of the resultant material [on silica, eluting with ethyl acetate / petrol / triethylamine (49:49:2)] only isolated the original product (16%, assuming monosilylated) and starting material, **99** (43%). Subsequent ^{1}H NMR spectroscopy ($CDCl_3$) indicated that the isolated product had decomposed, but confirmed the presence of the TBDMS group in the sample (by a series of signals below δ_H = 1ppm). TLC confirmed the presence of starting material, **99**, in the recovered NMR sample. Thus this isolated product remains unidentified, but is assumed to be a silylated derivative of Boc-Glyψ(NHCO)Gly-OEt, **99**, due to its reversion thereto and the accompanying silicon compound musty smell. So it is not clear which of the possible products was formed and / or isolated. Due to the instability of the product, and the low yield, this approach to solubilisation was not pursued.

(d) The influence of amino acid composition

Despite the failure of (most of) the attempted strategies, the fact that the desired chain extension involves incorporation of proline may weigh in favour of solubilisation. Proline has a very low propensity for incorporation into β-sheets,[187,204,271,276] and has been shown to break up the β-structure of otherwise troublesome peptide sequences.[272,286,323] Thus, the incorporation of proline during the elongation of the

PMRI tetrapeptides **104** and **122** was expected to at least stem the tide of decreasing solubility, and, in conjunction with the use of polar solvents, enable further studies in solution.

2.2 *Elongation of the PMRI Tetrapeptides, Boc-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-OEt, 104 and 122.-*

Formal chain extension of PMRI tetrapeptides **104** and **122** was examined concurrently by solution phase synthesis and SPS, the ultimate targets being PG^1-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-PG^2, where PG^1 = Boc and PG^2 = OEt (*e.g.* **140**, Xaa = Gly) or PG^1 = Ac and PG^2 = NH_2 (*e.g.* **146**, Xaa = Gly), respectively.

In addition, the synthesis of truncated products, Boc-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-OEt, **132** (Xaa = Gly) and **1** (Xaa = Val), was undertaken by solution phase methods only. These PMRI hexapeptides, **132** and **1**, were targeted because they retain the key pattern of peptide bond reversal found in the ultimate targets, *i.e.* every other peptide bond is reversed, except for the Pro-Gly bond in the turn region. So, the PMRI hexapeptides, **132** and **1**, have their first and fifth peptide bonds reversed, whereas the ultimate targets (*e.g.* **140**) have their first, third, seventh, and ninth peptide bonds reversed. The PMRI hexapeptides, **132** and **1**, are simply truncated versions of the ultimate target PMRI decapeptides, and as such are able to fold up in a similar fashion. Thus, the PMRI hexapeptides, **132** and **1**, represent pertinent targets for two reasons: (1) they are interesting molecules in their own right and for comparison with ultimate target PMRI decapeptides (*e.g.* **140**); and (2) they are synthetic intermediates on an alternative route to the ultimate target PMRI decapeptides (*e.g.* **140**), which may delay the onset of insolubility and the resultant problems thereof.

2.2.1 *Solution phase synthesis.*

(a) The truncated targets, 132 and 1

The strategy for the synthesis of the truncated products, **132** and **1**, was to minimise the number of steps that included the relatively inaccessible Xaaψ(NHCO)Gly units. Thus, a fragment synthesis approach was adopted: the three fragments being 2 x

[Xaaψ(NHCO)Gly] and the readily accessible Pro-Gly. The order of fragment coupling was designed to allow the use of a mixed anhydride coupling for the last step, thereby furnishing the desired products, **132** and **1**, relatively free from by-products and thus easing their purification.

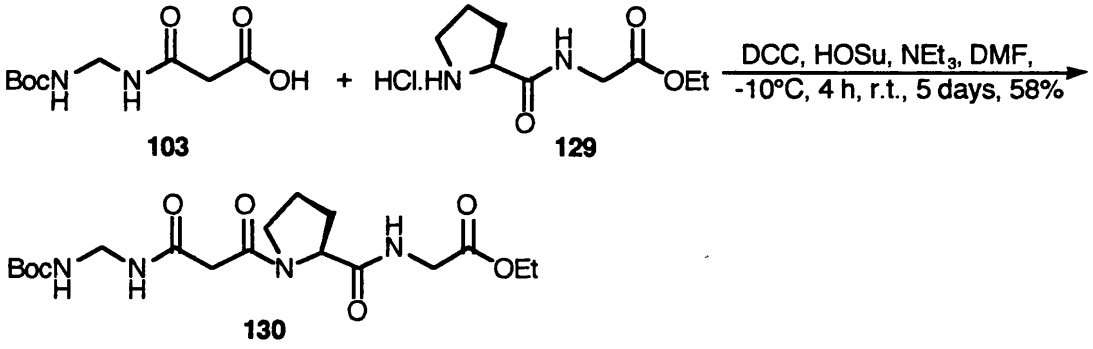
(i) Synthesis of HCl.Pro-Gly-OEt, 129

A standard DCC / HOBt mediated coupling of Boc-Pro and HCl.Gly-OEt provided Boc-Pro-Gly-OEt in *ca.* 75% yield. *N*-Terminal deprotection with ethanolic hydrogen chloride yielded HCl.Pro-Gly-OEt, **129** (91%).

(ii) Synthesis of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, 132

(1) Coupling of Boc-Glyψ(NHCO)Gly, 103, and HCl.Pro-Gly-OEt, 129

Boc-Glyψ(NHCO)Gly, **103**, and HCl.Pro-Gly-OEt, **129**, were coupled under standard DCC / HOSu conditions to yield Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130**, which was isolated by extraction with dichloromethane: scheme 66. Use of DMF, rather than THF, as solvent gave a better yield (58% vs 37%), due to improved dissolution of HCl.Pro-Gly-OEt, **129**.



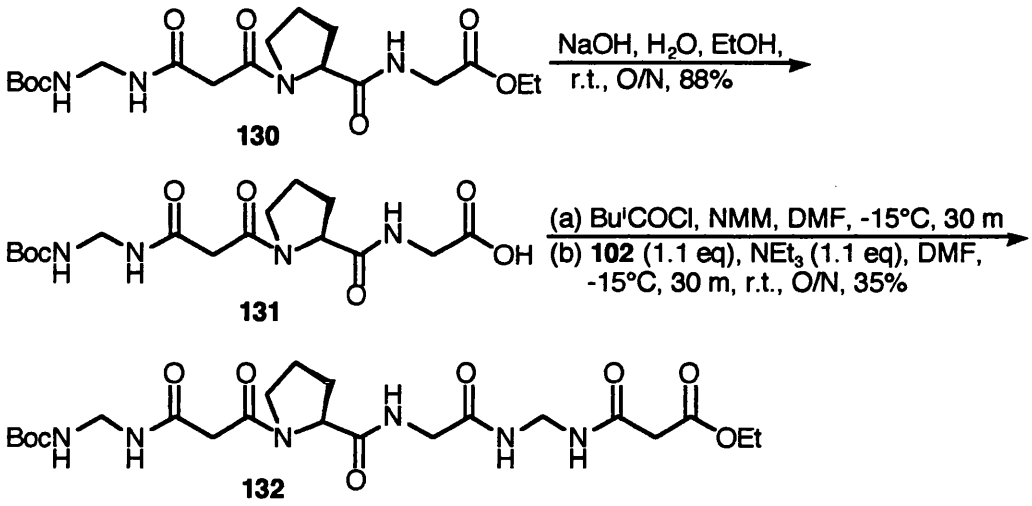
103 **129** **130**

Scheme 66: Coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Pro-Gly-OEt, **129**.

(2) C-Terminal deprotection and elongation of Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, 130

Saponification of Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130**, gave the free acid, **131**, in 88% yield, scheme 67. For the final coupling to TFA.Glyψ(NHCO)Gly-OEt, **102**, a mixed anhydride procedure was employed without fear of the previously experienced side reaction [see sections **2.1.1***(d)(ii)* and *(iii)(3)*] because here the *C*-terminal residue was not *m*Gly but plain Gly and therefore not susceptible. Thus, an *iso*-butyl carbonate mixed anhydride coupling of Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**, and

TFA.Glyψ(NHCO)Gly-OEt, **102**, under standard conditions,[242,251] provided Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**: scheme 67.



130 **131** **132**

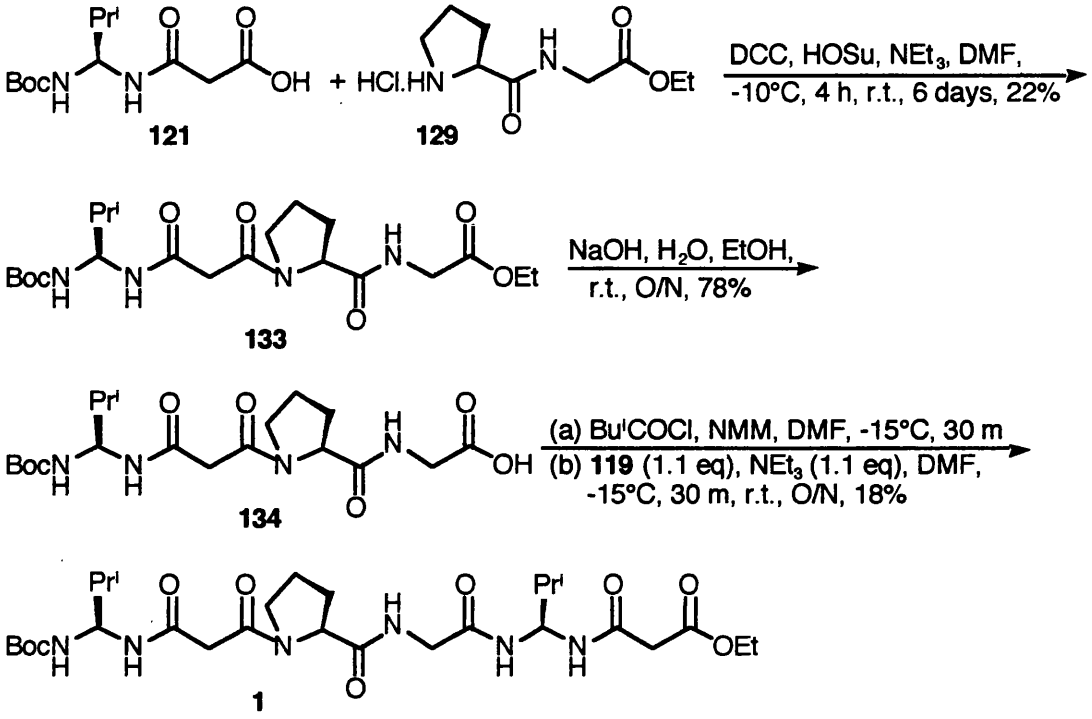
Scheme 67: *C*-Terminal deprotection and elongation of Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130**.

The product, **132**, proved to be insufficiently soluble in dichloromethane for its efficient extraction, despite the inclusion of proline in the middle of its sequence [*c.f.* section **2.1.4***(d)*]. Therefore it was isolated using a work-up procedure similar to that developed for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** (see experimental chapter). Column chromatography of the resultant crude product provided pure Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**, in 35% yield.

It is interesting that the yield of this coupling reaction was less than that of the HOSu active ester route to Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** [see section **2.1.1***(d)(iii)(2)*]. In the present case none of the previously discussed side reactions involving DCC [see section **2.1.1***(d)*] are possible, only those that stem from decomposition of the amino component, **102**. This suggests that the moderate yields obtained in couplings to *gem*-diamino residues are primarily due to decomposition [a problem exacerbated by inherently slow couplings to malonyl residues: see section **1.5.1***(a)(i)(1)*]. This problem is augmented by difficult isolation and purification for most of the products investigated in this project. No side reactions were observed (*c.f.* reference 100) and the only by-product identified was unreacted Boc-Glyψ(NHCO)Gly-Pro-Gly, **131** (by TLC).

(iii) Synthesis of Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, 1

Much the same approach furnished Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1**: scheme 68.



121 **129** **133** **134** **1**

Scheme 68: Synthesis of Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1**.

As in the corresponding Gly series, the PMRI tetrapeptide, **133** (but not the PMRI hexapeptide, **1**), was readily isolated by extraction with dichloromethane.

The lower coupling yields, with respect to those leading to **130** and **132**, are not surprising in light of the previous experiences *en route* to Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122** [see section **2.1.2***(d)*]. Indeed, ethyl malonamate, **118**, was isolated in 19% yield from the coupling reaction of Boc-Valψ(NHCO)Gly-Pro-Gly, **134**, and TFA.Valψ(NHCO)Gly-OEt, **119**, confirming that decomposition of **119** is at least partially accountable for the low yield of this step. No other by-products were identified in this sequence of reactions, except for unreacted starting materials, **121** and **134** (by TLC), in their respective coupling steps.

*(b) Attempted synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **140***

Solution phase synthesis of the ultimate targets was only attempted for Xaa = Gly, **140**.

Given that the truncated product Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**, did not display high solubility, it was not used as an intermediate *en route* to PMRI decapeptide, **140**. Rather, a fragment coupling strategy, with the minimum number of coupling steps, was adopted. Thus the strategy was to couple two Glyψ(NHCO)Gly-Glyψ(NHCO)Gly fragments to Pro-Gly, rather than extend **132** at its carboxy and amino termini (*i.e.* three coupling steps featuring Glyψ(NHCO)Gly moieties rather than four). The coupling to the *N*-terminus of the Pro-Gly fragment was expected to be sterically hindered and was therefore tackled first.

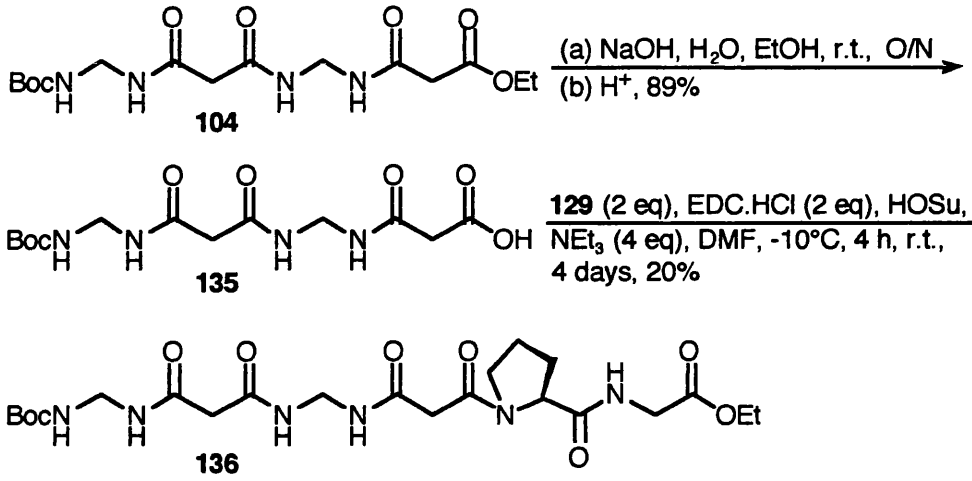
*(i) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104***

Saponification of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, gave the free acid, **135** scheme 69, though not without a minor complication. Acidification of the reaction mixture with 1 M potassium hydrogen sulfate, as used in the syntheses of acids **103**, **131**, *etc*, lead to precipitation. Attempts to isolate and wash this precipitate (with water or dilute acetic acid) merely resulted in its dissolution. Therefore the desired acid, **135**, was isolated by freeze-drying the aqueous reaction mixture followed by column chromatography [with chloroform / methanol / acetic acid (70:28:2) as eluent] of the resultant colourless solid.[¶] This procedure was unsatisfactory. Therefore an alternative acidification protocol was adopted. Acidification of the reaction mixture using the ion exchange resin Amberlite IR 120(+), decantation of the supernatant liquid (along with the suspended solid) and freeze-drying, provided acid **135** in 89% yield without resorting to chromatography.

[¶] Given that Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, was only sparingly soluble in dichloromethane, an organic extraction (as used for acids **103**, **131**, *etc.*) was expected and found to fail.

(ii) Synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, 136

In contrast to the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, there is no advantage to be gained by isolation of an active ester *en route* to Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**; for its precursor, **135**, already exhibits poor solubility. Nevertheless, the lessons learned during the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** [*c.f.* section **2.1.1***(d)*], were useful and prompted the choice of EDC / HOSu as the coupling procedure: scheme 69.



Scheme 69: Synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**.

Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, like its sequential isomer, Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**, proved to be insufficiently soluble in dichloromethane to permit its isolation by extraction, despite the β-structure disrupting potential of proline [see section **2.1.4***(d)*]. Neither was it possible to follow the protocol developed for isolation of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**: very little precipitation occurred upon addition of water to the evaporated reaction mixture. Therefore PMRI hexapeptide **136**, was isolated by column chromatography without any prior clean-up. Thus, PMRI hexapeptide **136**, was obtained in 20% yield.

This yield was rather disappointing in comparison with the analogous coupling leading to Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130** [58%: see section **2.2.1***(a)(ii)(1)*]. No by-products were identified, other than Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly, **135** (by TLC). Presumably the characteristically poor coupling of malonyl residues [see sections **1.5.1***(a)(i)(1)*, **2.1.1***(d)* and **2.1.2***(d)*], augmented by isolation and purification difficulties,

are to blame. Additionally, the fact that the coupling was to sterically hindered proline probably contributed to the low yield: side reactions such as ring opening of the intermediate HOSu ester, Pro-Gly diketopiperazine formation or *N*-acyl urea formation are known under these circumstances,[100] although there is no direct evidence that these side-reactions occurred in this case [or in any of the couplings to HCl.Pro-Gly-OEt, **129** undertaken].

Saponification of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, followed by acidification with Amberlite IR 120(+), provided Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly, **138**, in 84% yield, scheme 71.

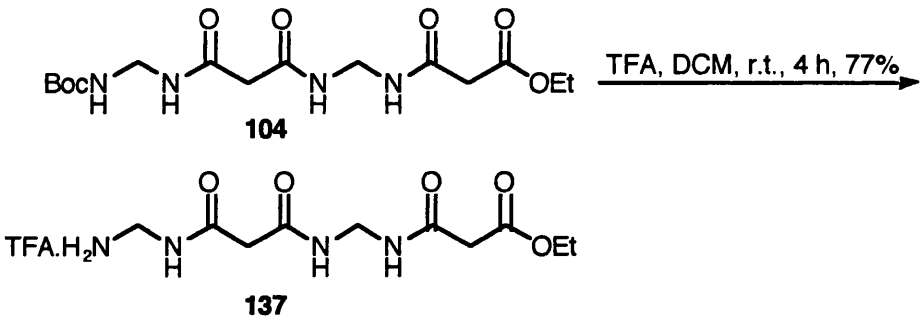
(iii) N-*Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104***

(1) Deprotection using hydrogen chloride

N-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, was initially performed using ethanolic hydrogen chloride and produced HCl.Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **139**, in quantitative crude yield, but with some decomposition (indicated by ^{1}H NMR spectroscopy).

(2) Deprotection using TFA

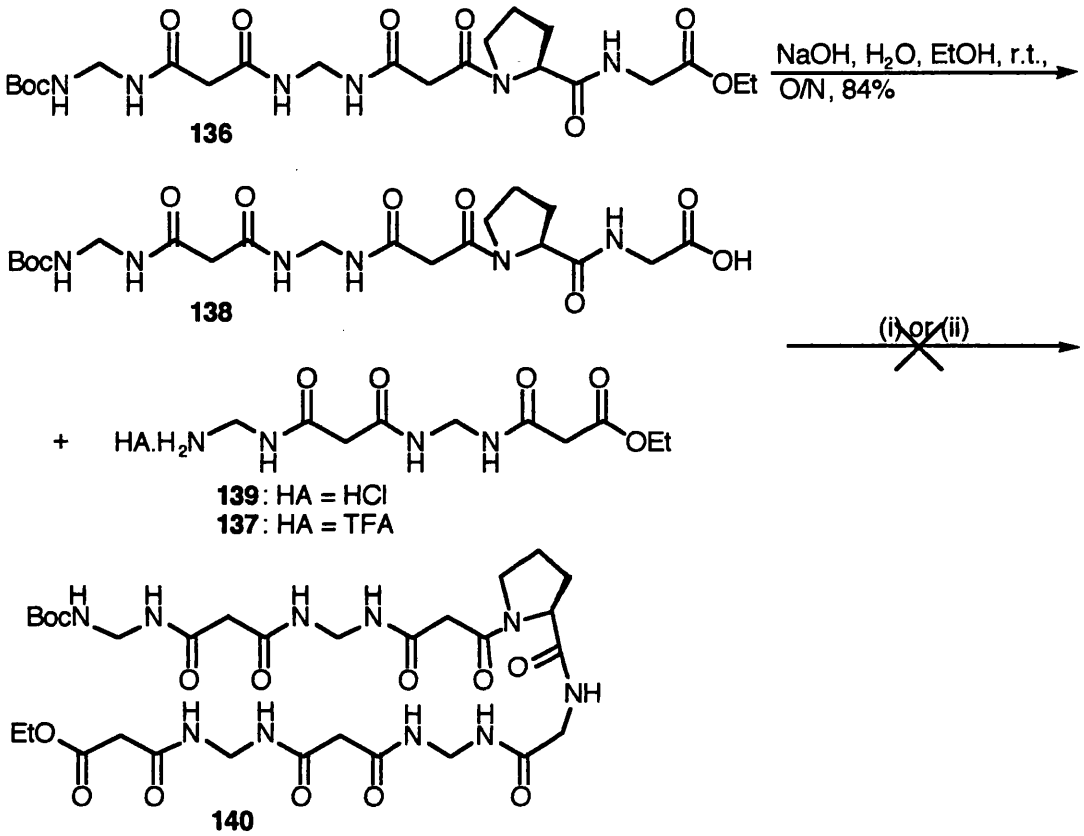
In view of the decomposition problems generally experienced during *N*-Boc deprotection using hydrogen chloride [sections **2.1.1***(b)(i)*, **2.1.2***(b)(i)* and **2.1.3***(b)(i)*], TFA was used in preference for the *N*-Boc deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**. Thus TFA.Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **137** of reasonable purity was obtained in 77% yield: scheme 70.



Scheme 70: *N*-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, with TFA.

*(iv) Attempted coupling of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly, **138**, and HA.Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt*

Two procedures that had previously proved useful in comparable coupling reactions were attempted for this step: EDC / HOSu and *iso*-butyl carbonic anhydride couplings. The desired reaction is depicted in scheme 71, but neither coupling procedure yielded any PMRI decapeptide, Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **140**.



Scheme 71: *C*-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, and attempted elongation of the resultant free acid, **138**. (i) **139**, EDC.HCl, HOSu, NEt_3 (2 eq), DMF, -5°C, 4 h, r.t., 9 days. (ii) (a) Bu^iOCOCl, NMM, DMF, -15°C, 30 m; (b) **137** (1.1 eq), NEt_3, DMF, -15°C, 30 m, r.t., O/N.

In both cases investigation of the reactions' progress by TLC provided little information [even with chloroform / methanol / TFE / acetic acid (85:10:3:2) as eluent]. In the former case, scheme 71 step (i), product isolation following the protocol developed for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** (*i.e.* evaporation of the reaction mixture, addition of water and collection of the resultant precipitate), followed by column chromatography [on silica, with chloroform / methanol / acetic acid (95:3:2 increasing to

75:20:5 and ultimately 0:90:10)] failed to isolate any of the desired PMRI decapeptide, **140**. In the latter case, scheme 71 step (ii), the residue from evaporation of the reaction mixture was chromatographed on Sephadex G25, with 1 M lithium chloride in THF as eluent,[293] but again none of the desired PMRI decapeptide, **140**, was isolated. Furthermore in both cases no identifiable material whatsoever was isolated.

It is thus unclear whether any of the desired PMRI decapeptide, **140**, was formed. In view of the experience with its precursors, Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **140**, was certainly expected to be highly insoluble and thus difficult to isolate, but any further analysis of the failure of these abortive couplings would be merely speculative.

2.2.2 *Solid phase synthesis.*

(a) Strategy

The guiding principles in our choice of a SPS strategy were: (1) to use, as much as possible, the knowledge and methods arrived at during the solution phase synthesis; and (2) to use the expertise and facilities within the department. Therefore, rather than form the *gem*-diamino residues on the solid support [*i.e.* by use of IBTFA, see section **1.5.1***(b)(i)*], the plan was to incorporate them within PMRI dipeptide building blocks (hereafter referred to as the monomers).

This decision posed the question of *N*-terminal protecting group choice, the options being Boc, Fmoc or MNP. Fmoc was chosen because it was for this protecting group that the in-house expertise and facilities were available. Furthermore, use of the Fmoc group, in conjunction with a dye, under continuous flow SPS conditions, permits monitoring of the progress of deprotection and acylation steps using post- and pre-column UV absorption measurement respectively.[324-326]

Previous studies of the SPS of PMRI peptides [see section **1.5.1***(b)*] and our solution phase synthesis results suggested three areas of potential difficulty, which we planned to tackle as follows.

*(i) Troublesome anchorage of malonyl residues [see section **1.5.1**(b)(iii)]*

As a free carboxy-terminus in the target PMRI decapeptide was not required, this potential problem could be avoided by use of the PAL-PEG-PS support.[327] PAL anchors the (PMRI) peptide to the solid support through an amide bond and was thus expected not to suffer from the difficulties encountered by Verdini and co-workers with their anchorage by malonyl residue esterification.[165] On ultimate cleavage from the resin, the PAL handle yields a (PMRI) peptide amide,[327] which is a suitable form of *C*-terminal blocking for the purposes of this study.

(ii) Slow couplings

Slow couplings, especially between malonyl and *gem*-diamino residues, were expected from previous results, therefore we planned to manually prolong these steps until the UV monitoring indicated their completion. The coupling procedure chosen was DIPCDI / HOBt, which is frequently employed in Fmoc continuous flow SPS. This method was chosen because carbodiimide couplings were successful in the solution phase studies and the nature of SPS makes those previously experienced isolation and purification difficulties specific to carbodiimides [see section **2.1.1***(d)(i)*] irrelevant.

Standard incorporation of Gly^6 and Pro^5 as their Fmoc-Xaa-OPfp derivatives was anticipated to proceed without complications.

(iii) Decomposition of free gem*-diamino residues*

In order to minimise the opportunity for decomposition of the deprotected *gem*-diamino residues [*c.f.* section **1.5.1***(a)(i)(5)*], the automated, shortened, "diketopiperazine deprotection protocol" was selected.

(iv) A "difficult sequence"?

Given the aggregation problems experienced during solution phase synthesis, there was the possibility that the SPS would become that of a "difficult sequence". UV monitoring of the *N*-Fmoc deprotection peak profiles would enable assessment of this situation and thus permit extension of deprotection and subsequent coupling times if necessary.[282,324]

If this problem was to become too grave then one of the previously investigated solubilisation strategies could be employed, *e.g.* resin swelling using TFE, HFIP or a lithium chloride solution [see section **2.1.4***(a)*].

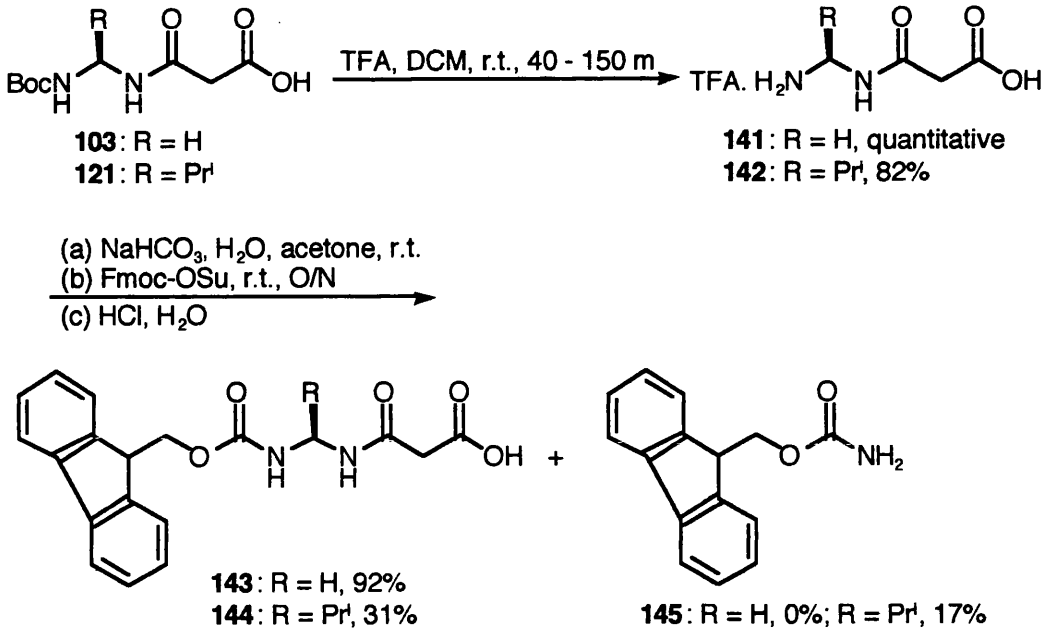
*(b) Synthesis of the monomers, Fmoc-Xaaψ(NHCO)Gly, **143** (Xaa = Gly) and **144** (Xaa = Val)*

The Fmoc group was designed to be cleaved under mild basic conditions.[325,328] It is therefore highly unlikely that the required *N*-Fmoc SPS monomers, **143** and **144**, could be synthesised from Fmoc-Xaa using our Goldschmidt and Wick type procedure (schemes 40 and 52). The alternative is to transform Boc-Xaaψ(NHCO)Gly into Fmoc-Xaaψ(NHCO)Gly. Although simple reaction sequences (and even one-pot transformations) exist for *N*-Boc to *N*-Z,[322] *N*-Z to *N*-Boc,[329,330] *N*-Fmoc to *N*-Z,[331] and *N*-Fmoc to *N*-Boc,[332] there is no such procedure for *N*-Boc to *N*-Fmoc. It was therefore necessary to effect this transformation in two steps.

*(i) C-Terminal deprotection of Boc-Xaaψ(NHCO)Gly, **103** (Xaa = Gly) and **121** (Xaa = Val), and subsequent introduction of Fmoc*

Treatment of Boc-Xaaψ(NHCO)Gly, **103** and **121**, with TFA / dichloromethane followed by the previously developed work-up procedure (see experimental chapter) yielded the corresponding fully deprotected PMRI dipeptides, **141** and **142**: scheme 72. Neither of these products, **141** and **142**, showed any trace of ammonium trifluoroacetate (by ^{1}H NMR spectroscopy), although TFA.Valψ(NHCO)Gly, **142**, was contaminated by traces of by-products [one of which could be malonamic acid (as indicated by ^{1}H and ^{13}C NMR spectroscopy)]. An attempt to purify TFA.Valψ(NHCO)Gly, **142**, by crystallisation from ethanol was unsuccessful. It is not obvious why Boc-Xaaψ(NHCO)Gly, **103** and **121**, should suffer less decomposition during *N*-Boc deprotection than the corresponding ethyl esters, **99** and **116(a)** [*c.f.* sections **2.1.1***(b)(ii)* and **2.1.2***(b)(ii)*].

There is a selection of commercially available reagents that introduce the Fmoc group: Fmoc-Cl, Fmoc-N_3 and Fmoc-OSu.[325] Fmoc-OSu is reputedly the best reagent with regard to product purity,[325] so it was employed, according to Paquet's procedure,[333] to prepare Fmoc-Xaaψ(NHCO)Gly, **143** and **144**: scheme 72.



Scheme 72: Preparation of the SPS monomers, **143** and **144**.

For Fmoc-Glyψ(NHCO)Gly, **143**, this synthesis was very successful, despite the failure of the literature work-up procedures[333,334] because the product was insoluble in chloroform (or dichloromethane or ethyl acetate) precluding its extraction. Instead, Fmoc-Glyψ(NHCO)Gly, **143**, was isolated by washing the precipitated crude product with water, and purified by column chromatography. The resultant material was contaminated with traces of HOSu,* but used without further purification as HOSu would not interfere with the SPS.

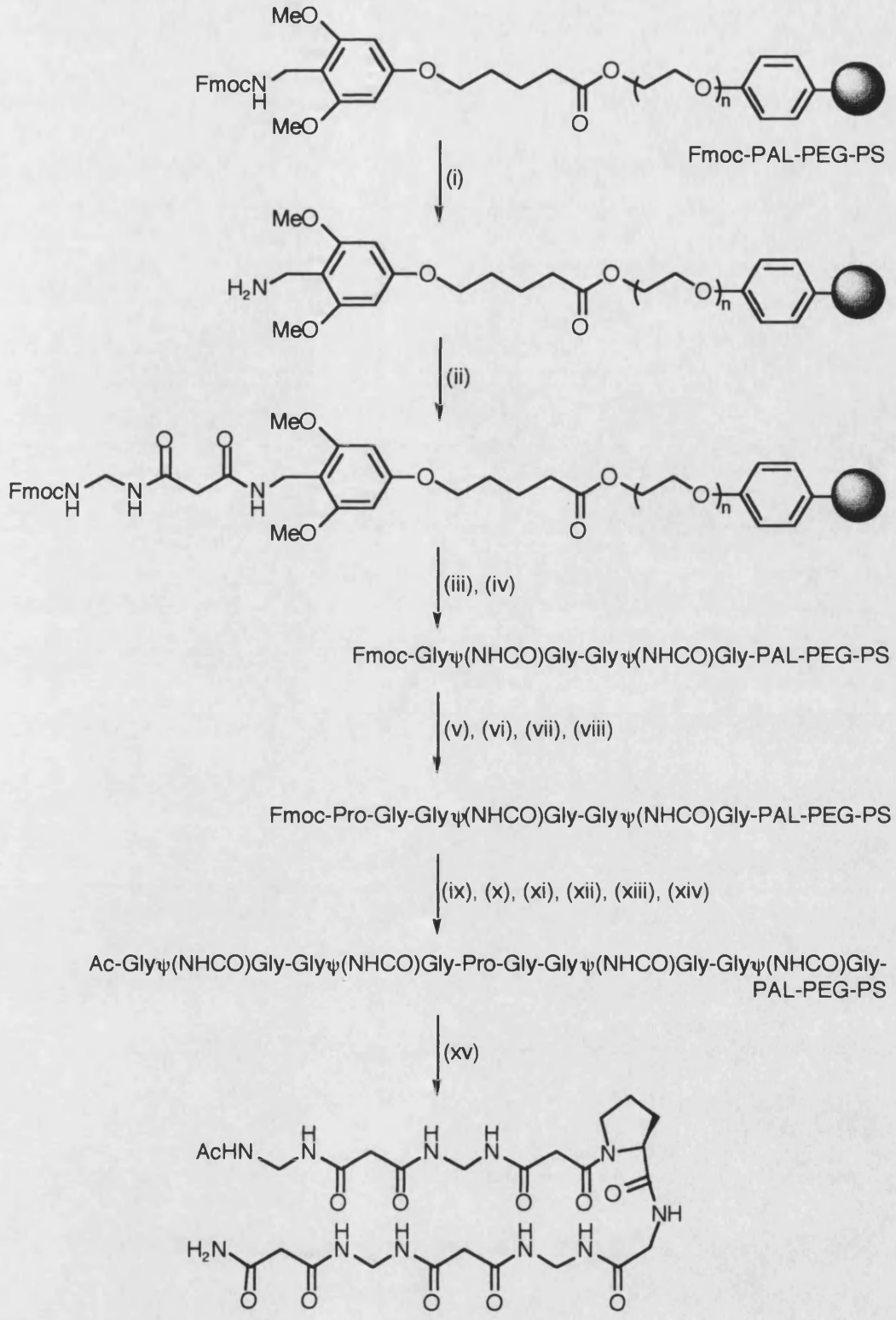
Similarly the literature work-up procedures[333,334] failed for Fmoc-Valψ(NHCO)Gly, **144**, so the method developed for the isolation and purification of Fmoc-Glyψ(NHCO)Gly, **143** was applied. After column chromatography Fmoc-Valψ(NHCO)Gly, **144** was again contaminated with HOSu (crude yield 50%), which was removed by crystallisation from acetone / water to provide Fmoc-Valψ(NHCO)Gly, **144**, in 31% yield. Also isolated from the synthesis of Fmoc-Valψ(NHCO)Gly, **144** was 9-fluorenylmethyl carbamate (Fmoc-NH_2), **145**, which derives from the decomposition of

* ^{1}H NMR spectroscopy indicated that the ratio of Fmoc-Glyψ(NHCO)Gly, **143** to HOSu was 94:6; therefore the corrected yield of Fmoc-Glyψ(NHCO)Gly, **143** is 90%. Crystallisation from ethanol gave pure Fmoc-Glyψ(NHCO)Gly, **143** containing retained ethanol, which certainly would interfere with the SPS.

TFA.Valψ(NHCO)Gly, **142** under (or prior to) the conditions of the Fmoc introduction. There was no sign of this by-product in the synthesis of Fmoc-Glyψ(NHCO)Gly, **143**, presumably because the absence of a side chain makes TFA.Glyψ(NHCO)Gly, **141** more resistant to decomposition [the *iso*-propyl side chain of TFA.Valψ(NHCO)Gly, **142** stabilises the intermediate iminium species on the decomposition pathway: *c.f.* sections **1.5.1***(a)(i)(5)* and **2.1.2***(b)(i)*]. Alternative methods of Fmoc introduction[325] might improve the yield of Fmoc-Valψ(NHCO)Gly, **144**, but none were attempted. Furthermore, the low yield of this reaction meant that there was insufficient Fmoc-Valψ(NHCO)Gly, **144** to proceed with the SPS of Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt.

*(c) SPS of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH$_2$, **146***

As planned, the SPS of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146** was performed on a PAL-PEG-PS[327] support (on a 0.1 mmol scale using a fourfold excess of coupling reagents). The coupling steps were monitored by following the displacement of a dye (acid violet 17) from the resin by the acylating species (by measuring the pre-column UV absorption; this technique is called counterion distribution monitoring).[326] The couplings to malonyl residues [*i.e.* steps (ii), (iv), (x) and (xii), scheme 73] were prolonged manually until the pre-column detector recorded a steady maximum, indicating no further acylation. These steps typically took *ca.* 24 h. except for the last coupling [step (xii), scheme 73], which was slower. Gly^6 and Pro^5 were successfully incorporated, under automatic control, as their commercially available Fmoc-Xaa-OPfp derivatives [steps (vi) and (viii), scheme 73], requiring coupling times of 3 h and 1 h respectively. No problems associated with steric hindrance due to proline were manifest.



146

Scheme 73: SPS of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146**. (i) Pip, DMF. (ii) **143**, DIPCDI, HOBt, DMF. (iii) As (i). (iv) As (ii). (v) As (i). (vi) Fmoc-Gly-OPfp, HOBt, DMF. (vii) As (i). (viii) Fmoc-Pro-OPfp, HOBt, DMF. (ix) As (i). (x) As (ii). (xi) As (i). (xii) As (ii). (xiii) As (i). (xiv) Ac_2O, HOBt, DMF. (xv) TFA, H_2O, r.t., 2 h.

The Fmoc deprotection steps [(i), (iii), (v), (vii), (ix), (xi) and (xiii), scheme 73] were also monitored (by measuring the post-column UV absorption). The resultant deprotection profiles [those for steps (i) and (xiii), scheme 73 are depicted in figures A4(a) and (b)] indicated successful incorporation of each preceding residue {*e.g.* the existence of a deprotection profile for step (xiii), scheme 73 [figure A4(b)], indicated that $Gly^{1}\psi(NHCO)Gly^{2}$ was successfully incorporated}.[324,325] Moreover, none of the deprotection profiles displayed the distortion observed during the SPS of "difficult sequences", *i.e.* a flattening and broadening, indicating slowing of the deprotection rate.[282,324] As shown in figure A4, the first and last deprotection steps proceeded normally, despite the fact that the last coupling step [(xii), scheme 73] needed 48 h to reach its steady maximum pre-column detector reading.

Therefore the monitoring of both acylation and deprotection during the SPS indicated that each step proceeded satisfactorily, *i.e.* the resin bound PMRI decapeptide, **146** was successfully synthesised.

The PMRI decapeptide, **146** was cleaved from the resin using TFA / water (95:5), step (xv), scheme 73. No additional scavengers were included in the "cleavage cocktail" (*c.f.* reference 327) because no alkylation was expected as no temporary protecting groups were present that could give rise to alkylating species during the cleavage. Additionally no alkylation was observed during *N*-Boc deprotections using (scavenger free) TFA solutions, in the course of the solution phase synthesis [sections **2.1.1***(b)(ii)* and **2.2.1***(b)(iii)(2)*], indicating that such PMRI peptides are not overly susceptible to alkylation during acidoyltic deprotection. Furthermore, if any PMRI decapeptide, **146** were alkylated by the resin bound PAL-derived carbonium ion it would remain resin bound and thus not contaminate the product.† We were keen to avoid additional scavengers in order to circumvent the potentially difficult removal thereof.

The resultant crude PMRI decapeptide, **146** was insufficiently soluble in the standard HPLC solvent system to permit its analysis or purification. Therefore a portion

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† A test cleavage yielded crude PMRI decapeptide, **146**, without any sign of unforeseen complications, so the bulk of the material was cleaved in the same fashion.

of the material was flash column chromatographed on silica, eluting with chloroform / methanol / TFE / acetic acid (85:10:3:2).‡ However no PMRI decapeptide, **146** was recovered. The remaining crude material was successively washed and centrifuged, which effected some purification, although the low density of the material (a result of freeze-drying) caused low recovery.

Evidence for the successful SPS of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146** (in addition to that given by the UV monitoring during the assembly) is provided by an electrospray mass spectrum of the crude material (obtained despite solubility difficulties), which contains a $(M+Na)^+$ and $(M+H)^+$ peak. The mass spectrum's fragmentation pattern contains only one other assignable peak, but there are no peaks that correspond to deletion sequences {*i.e.* [**146** - a Glyψ(NHCO)Gly moiety + H]$^+$ = 556}. Neither are there any peaks appropriate for decomposition PMRI peptides [*i.e.* terminated due to *gem*-diamino residue decomposition resulting in, *e.g.* H_2N-*m*Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2; all the possible $(M+H)^+$ ions from this process (599, 485, 216 and 103) are absent]. ^1H NMR spectroscopy furnishes additional evidence. ^1H NMR spectra of the crude and washed material, accumulated in $DMSO_{d6}$, are consistent with the desired PMRI decapeptide, **146** (see experimental chapter). An attempt to accumulate ^1H NMR spectra in TFE / $methanol_{d4}$ (95:5), having applied the solubilising pretreatment of Tanner and co-workers (*i.e.* dissolution in TFA, followed by cycles of dilution with TFE and concentration)[335] failed. Although this NMR sample solution was initially clear it clouded on standing and the resultant spectrum was very poor. Spectra accumulated in lithium chloride / THF_{d8} were similarly useless: the crude PMRI decapeptide, **146** did not dissolve well in this mixture, possibly due to retained water.

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‡ A similar solvent system was successfully used to purify thymosin α_1 fragments.[285] Given that both a chloroform / methanol / acetic acid eluent with silica as the stationary phase and chromatography on Sephadex G25 with 1 M lithium chloride in THF as eluent failed for purification of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **140**, these approaches were not attempted for Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146**.

In summary, PMRI decapeptide, **146** was successfully synthesised by solid phase methods, but its low solubility thwarted effective purification and thus thorough characterisation and analysis.

2.3 *Conformational Studies.-*

Our initial plan was to study the conformations of our PMRI peptides principally using NMR techniques,[336-340] supplemented by X-ray crystallography[185,341,342] and, where applicable, CD spectroscopy.[187,343-345] However, the solubility difficulties that complicated the syntheses also caused problems in our conformational studies. As a result it was necessary to perform all the NMR studies on the PMRI peptides of interest (*i.e.* PMRI tripeptides and above) in $DMSO_{d6}$. As DMSO is a strong hydrogen bond accepting solvent it is likely to disrupt both intermolecular hydrogen bonding [hence effecting dissolution: *c.f.* section **2.1.4***(a)(i)*] and intramolecular hydrogen bonding (hence disrupting secondary structure).§ Furthermore the solubility difficulties meant that we were unable to obtain any data from X-ray crystallography.¶

Limited NMR studies were undertaken and are described below, along with a measurement of CD spectra of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**. Phase sensitive COSY and NOESY experiments were performed on the PMRI peptides with structure of interest. Results from these studies allowed the assignment of the ^1H NMR resonances of the PMRI peptides. In addition, VT ^1H NMR spectra were recorded and analysed to study the changes in the chemical shifts of the amide H-resonances. This was undertaken in order to assess hydrogen bonding within the PMRI peptides.[338] For all the PMRI peptides studied [except HCl.Glyψ(NHCO)Gly-OEt, **101**: see section **2.1.1***(b)(i)*] no decomposition was observed during the VT experiments. This was confirmed by recording a final ^1H NMR spectrum on cooling the sample to r.t. after

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§ However some (modified) peptides have been shown to form β-sheet type structures in DMSO, *e.g.* references 13,28,216,346 and 347.

¶ Of the PMRI peptides larger than dipeptides, only ethylmalonyl-Valψ(NHCO)Gly-OEt, **120**, was crystalline, but crystals of sufficient quality for X-ray crystallography were not obtained.

the VT experiment was complete. The discussion below is arranged in order of PMRI peptide length.

In theory IR spectroscopy could also have been employed. Indeed the peptide or protein β-sheet conformation exhibits a characteristic IR absorption pattern in the amide I region: a strong absorption at *ca.* 1625 cm^{-1}, with a shoulder at *ca.* 1695 cm^{-1} for the antiparallel β-sheet, and a single strong absorption at *ca.* 1635 cm^{-1} for the parallel β-sheet.[187,345,348,349] These absorption bands may be observed in solution or the solid state.

None of our PMRI peptides that might adopt β-sheet structure exhibited such absorptions in their IR spectra (see experimental chapter). However, it is by no means clear whether the β-sheet absorption bands exhibited by peptides and proteins should be displayed by PMRI peptides,* and furthermore Narita and co-workers demonstrated that the application of shear stress (as occurs during the preparation of Nujol mull samples) can disrupt β-sheet structure.[350,351] Therefore no conclusions concerning the existence of β-sheet structure can be drawn from the Nujol mull IR spectra of our PMRI peptides, and their low solubility precluded additional studies in solution.

2.3.1 *The PMRI tripeptide ethyl malonyl-Valψ(NHCO)Gly-OEt, 120.*

The NOESY spectrum of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120** contains only the cross peaks expected from its primary structure, figure 19, and none corresponding to a folded conformation. This is consistent with the expected extended structure of **120**, but the absence of NOEs cannot be taken as a positive indication of any particular structure.[352]

The scalar coupling constants (*J*s) observed in the ^1H NMR spectrum of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, similarly provide only ambiguous information. Literature precedent suggests that the usual Karplus-type equation [$^3J_{HNC\alpha H}$ = $A\cos^2(\phi\pm60) - B\cos(\phi\pm60) + C$ (+ for L-amino acids, - for D-amino acids) or

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* The only IR spectroscopic studies carried out on PMRI peptides are those of Gellman and co-workers [see section **1.5.3***(b)(iv)(2)*] and those of Marraud and co-workers [see section **1.5.3***(b)(i)*], which were performed in solution and required individual absorption assignment and / or suitable reference compounds.

$\Sigma(^3J_{HNC\alpha H_2}) = -A\cos^2\phi - B\cos\phi + C$ for glycine][336,353] used for amino acids and peptides is applicable to PMRI peptide *gem*-diaminoalkyl residues (the D-amino acid version being used with $^3J_{HC\alpha\psi NH}$ to determine ψ).[172,354,355,†] For ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, the value of $^3J_{HNC\alpha H}$ (8.4 Hz) is indicative of (ϕ,ψ) = (-150°,150°), (-90°,90°), or (40°, -40°) to (80°,-80°), using Bystrov's Karplus curve.[‡,353] This data therefore permits no firm deductions regarding the conformation of ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**, especially considering the proximity of the observed value of $^3J_{HNC\alpha H}$ to the average value for a freely rotating bond (7.5 Hz),[338,356] and the similar value recorded for Boc-Valψ(NHCO)Gly-OEt, **116(a)**.

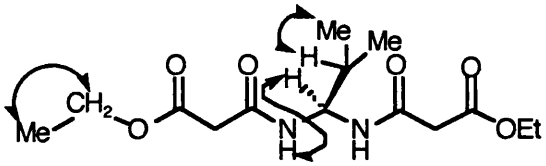

Figure 19: NOEs observed for ethyl malonyl-Valψ(NHCO)Gly-OEt, **120**; NOEs observed between multiple symmetrically identical protons are only indicated once (in this and subsequent figures).

Measurement of the variation of the NH chemical shifts with temperature for PMRI tripeptide **120**, produced a linear relationship (over the range 20-100°C) with gradient -5×10^{-3} ppm/K, indicative of a solvent exposed NH, confirming the absence of intramolecular hydrogen bonding.[338]

Therefore all these NMR experiments on ethyl malonyl-Valψ(NHCO)Gly-OEt, **120** provided no strong evidence for any particular secondary structure, but conversely, provided no evidence inconsistent with the expected extended structure.

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† Use of $^2J_{HC\alpha H}$ to determine the (ϕ,ψ) of *m*Gly residues is, conversely, unprecedented. By comparison with native peptides a relationship is expected,[338,353] but it is unclear what form it might take in PMRI peptides and therefore no attempt has been made to utilise it in this study.

‡ Bystrov's Karplus curves were also used in all subsequent *J* analysis. The resultant values of (ϕ,ψ) are only approximate.

2.3.2 *PMRI tetrapeptides.*

(a) Boc-Xaaψ(NHCO)Gly-Xaaψ(NHCO)Gly-OEt, ***104*** *(Xaa = Gly) and* ***122*** *(Xaa = Val)*

The NOESY spectra of **104** and **122** contain only cross peaks indicative of primary structure: figures 20 and 21.

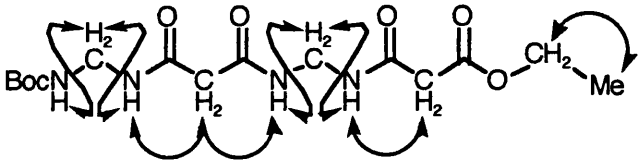

Figure 20: NOEs observed for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**.

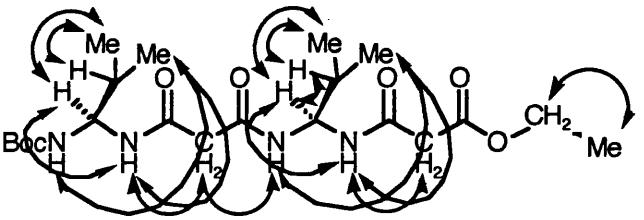

Figure 21: NOEs observed for Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**.

For Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, the values of $\Sigma(^3J_{HNC\alpha H_2})$ indicate the ambiguous set of (ϕ,ψ) values shown in table 3.

Table 3:

	$\Sigma(^3J_{HNC\alpha H_2})$	ϕ / °	$\Sigma(^3J_{H_2C\alpha\psi NH})$	ψ / °
Gly[1]	11.6	±130, ±50	12.0	±120, ±60
Gly[3]	11.0	±120, ±60	12.0	±120, ±60

For Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, the values of $^3J_{HNC\alpha H_2}$ indicate the ambiguous set of (ϕ,ψ) values shown in table 4.

Table 4:

	$^3J_{HNC\alpha H}$ / Hz	ϕ / °	$^3J_{HC\alpha\psi NH}$ / Hz	ψ / °
Val[1]	9.0	-130, -90, 50 to 70	8.4	150, 90, -40 to -80
Val[3]	8.1	-150, -90, 40 to 80	8.5	150, 90, -40 to -80

So, for both PMRI tetrapeptides **104** and **122**, as for PMRI tripeptide **120**, the NMR data does not indicate a folded structure and is consistent with, yet insufficient to prove, the expected extended (or any other rigid) structure.

*(b) Boc-Xaaψ(NHCO)Gly-Pro-Gly-OEt, **130** (Xaa = Gly) and **133** (Xaa = Val)*

No detailed study of these two PMRI tetrapeptides was undertaken, but brief comparisons between them (plus Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**) and longer PMRI peptides are made below.

2.3.3 *PMRI hexapeptides.*

*(a) Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136***

(i) Conformers

As is apparent from the NMR spectra of **136** (see experimental chapter), the incorporation of proline in its sequence gives rise to a major and minor conformer in DMSO solution. Indeed, this phenomenon was observed for all the PMRI peptides synthesised in this project that contain proline, *i.e.* **130**, **131**, **132**, **133**, **134**, **1**, **136** and **138**. This is a common observation for proline containing peptides: the two conformers differ in the configuration of the Xaa-Pro bond (*cis* vs *trans*), and equilibrate slowly on the NMR time scale.[338,357] The two conformers may be distinguished using the chemical shift difference between the proline Cβ and Cγ resonances in the ^{13}C NMR spectrum: a larger difference is observed for the *cis*-conformer.[338,357-360] Furthermore, the relative heights of the proline Cβ and Cγ peaks in the ^{13}C NMR spectrum may be used to determine the *cis* : *trans* ratio;[357] although in these investigations appropriate ^{1}H NMR integrals are employed to this end.

The relative intensities of the proline Cβ and Cγ ^{13}C NMR resonances (and / or the chemical shifts thereof) for all the proline containing PMRI peptides described herein indicate that the *trans*-conformer is the major form.

For Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136** the *cis* : *trans* ratio is *ca.* 30:70, *c.f. ca.* 36:64 for Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**. In the former case, acquisition of the ^{1}H NMR spectrum at 50°C did not lead to coalescence of the signals corresponding to the major and minor conformers. In the latter case a more extensive VT ^{1}H NMR study was carried out, and by 100°C, the major and minor signals

of both the BocNH and Gly$^{1}\psi$NH§ had coalesced, but those of Gly^{4}NH§, Gly$^{2}C^{\alpha}H_2$ and Pro$^{3}C^{\alpha}$H had not: presumably, therefore, rapid equilibration of the two conformers did not occur, even at 100°C. Figure 22 displays the variation observed in the NH chemical shifts with temperature for Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**. The temperature gradients for the NH chemical shifts are presented in table 5.

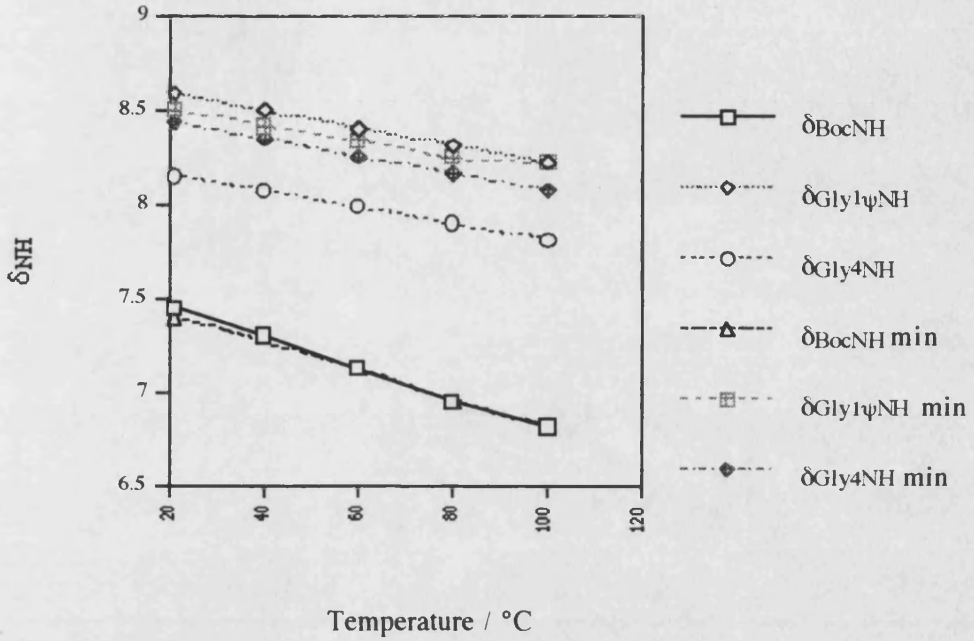

Figure 22: Variation of NH chemical shifts with temperature for Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**; min refers to the minor conformer.

Table 5:

	BocNH	Gly $^{1}\psi$NH	Gly ^{4}NH
$\frac{d\delta}{dT}$ / -10^{-3} ppm/K	8 (8)	5 (4¶)	4 (5)

The figures in brackets refer to the minor conformer.

¶ Significant deviation from linearity.

§ Tentatively assigned by comparison with **136**.

All these values are indicative of solvent exposed NHs, except that for Gly^{4}NH (major conformer), which is intermediate in value and thus inconclusive.[338,*]

*(ii) Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136** NOESY and J analysis*

The NOESY spectrum of **136**, in common with those described above, contains only (weak) cross peaks (observed for the major conformer only) corresponding to its primary structure: figure 23.

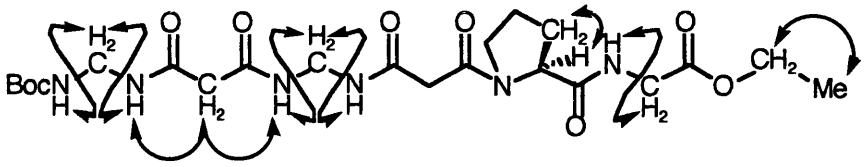

Figure 23: NOEs observed for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**.

The values of $\Sigma(^3J_{HNC\alpha H_2})$ and $^2J_{HC\alpha H}$ for Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, and the ambiguous set of (ϕ,ψ) values they indicate are shown in table 6.

Table 6:

	$\Sigma(^3J_{HNC\alpha H_2})$ / Hz	ϕ / °	$\Sigma(^3J_{H_2C\alpha\psi NH})$ / Hz	ψ / °
Gly^1	12.0	±120, ±60	br	
Gly^3	12.2	±120, ±60	12.0 (12.0)	±120, ±60
Gly^6	11.4 (11.0)	±130, ±50	17.4†	0 to ±30, ±150 to ±180

The figures in brackets correspond to the minor conformer.
† $^2J_{HC\alpha H}$

These (ϕ,ψ) values for the PMRI portion of **136** (*i.e.* Gly^1 and Gly^3) are very similar to those of **104**, again indicating no rigid secondary structure. Moreover, none of this NMR data is indicative of any (unexpected) folded structure.

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* For comparison, the values for Boc-Glyψ(NHCO)Gly-OEt, **99** (recorded in $DMSO_{d6}$) were: BocNH, -8×10^{-3} ppm/K and Gly$^1\psi$NH, -6×10^{-3} ppm/K.

(b) Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, 132

Unlike the preceding cases, PMRI hexapeptides **132** and **1** were expected to adopt (or at least populate) a rigid, folded (β-hairpin type) secondary structure.

(i) Conformers and VT ^{1}H NMR spectroscopy

As described above [section **2.3.3***(a)(i)*], PMRI hexapeptide **132** exists in *cis* and *trans*-conformers at -*m*Gly^2-Pro^3-: its *cis* : *trans* ratio, given by ^{1}H NMR spectroscopy, is *ca.* 34:66, much the same as that for PMRI peptides **131** and **136**. Therefore the desired β-hairpin type structure, if adopted, does not favour one conformer at the expense of the other. A β-hairpin type structure is only expected to be possible with a *trans* -*m*Gly^2-Pro^3- bond (in order to permit β-turn formation across residues -*m*Gly^2-Pro^3-Gly^4-*g*Gly^5-). Hence, this invariance of *cis* : *trans* ratio suggests that a β-hairpin type structure for PMRI hexapeptide **132** is either not significantly populated at all, or does not substantially stabilise the *trans*-conformer with respect to the *cis*.

The VT ^{1}H NMR study of PMRI hexapeptide **132** brought about only partial coalescence of the major and minor signals, as was the case for PMRI tetrapeptide **131** [see section **2.3.3***(a)(i)*], so rapid equilibration of the two conformers did not occur. Assignment of both the major and minor NH signals was possible using the COSY and NOESY results. The variation of the NH chemical shifts of PMRI hexapeptide **132** with temperature is plotted in figure 24, and the gradients shown in table 7. These values indicate exposure to solvent for BocNH, Gly^{1}ψNH, Gly^{5}NH, and Gly^{5}ψNH, whereas the value for Gly^{4}NH is ambiguous.[338] A β-hairpin type structure would result in solvent shielding for Gly^{5}NH, and Gly^{5}ψNH (due to hydrogen bonds between Gly^{2}CO and Gly^{5}NH, and Gly^{2}ψCO and Gly^{5}ψNH, *c.f.* figure 26), and exposure to solvent for the other NHs. Thus the VT ^{1}H NMR results do not indicate formation of a β-hairpin type structure by Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**. In contrast the 9-membered hydrogen bonded ring conformation characterised by Gellman and co-workers [see section **1.5.3***(b)(iv)(2)*] would result in solvent shielding of only Gly^{4}NH: the above VT results (and those for PMRI tetrapeptide **131**) may be consistent with such a structure, but the value of the NH chemical shift temperature gradient is ambiguous.

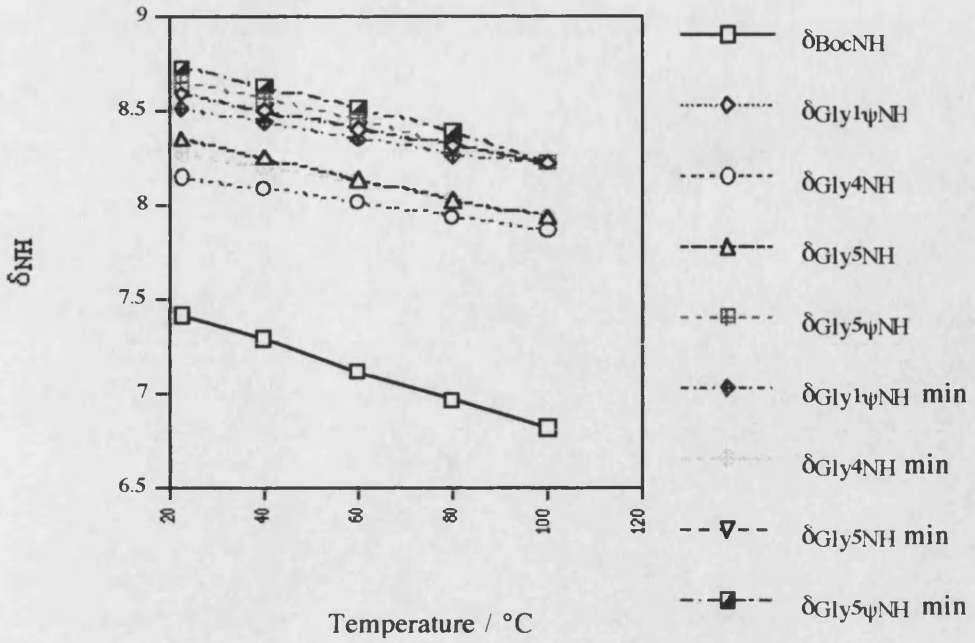


Figure 24: Variation of NH chemical shifts with temperature for Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**.

Table 7:

	BocNH	Gly ^{1}ψNH	Gly ^{4}NH	Gly ^{5}NH	Gly ^{5}ψNH
$\frac{d\delta}{dT}$ / -10^{-3} ppm/K	8	5(4‡)	4(4)	5(5)	6(6‡)

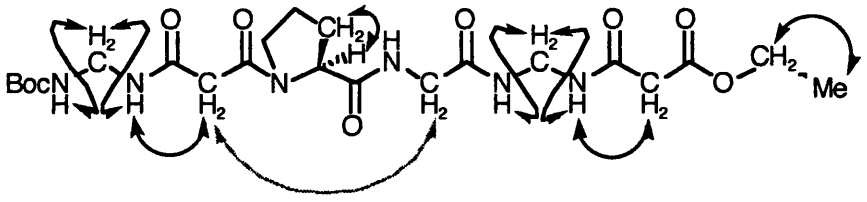
The figures in brackets refer to the minor conformer.

‡ Significant deviation from linearity.

(ii) NOESY and J *analysis*

The weak NOEs observed in the NOESY spectrum of PMRI hexapeptide **132**, for the major conformer, are depicted in figure 25.

Observation of the NOEs depicted in figure 26 would establish the adoption of a β-hairpin type conformation by Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**.[28,336,344,352]



= obscured by noise.

Figure 25: NOEs observed for the major conformer of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**.§

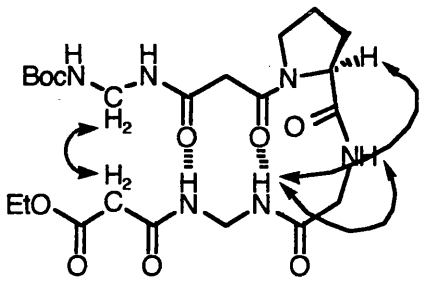


Figure 26: NOEs expected if Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132** adopted a β-hairpin type conformation.

None of these characteristic NOEs were observed. Rather, only NOEs indicative of primary structure were detected, except for a possible NOE between $Gly^2C^{\alpha}H_2$ and $Gly^5C^{\alpha}H_2$, but it is not clear whether this signal was an artefact or a real NOE.

The values of $\Sigma(^3J_{HNC^{\alpha}H_2})$ and $^2J_{HC^{\alpha}H}$ for PMRI hexapeptide **132**, and the ambiguous values of (ϕ,ψ) they imply are presented in table 8.

Table 8:

	$\Sigma(^3J_{HNC^{\alpha}H_2})$ / Hz	ϕ / °	$\Sigma(^3J_{H_2C^{\alpha}\psi NH})$ / Hz	ψ / °
Gly^1	br		12.8	±120, ±60
Gly^4	12.0 (12.0)	±130, ±60	16.7¶	0 to ±40, ±140 to ±180
Gly^5	12.0	±130, ±60	12.0 (11.6)	±130, ±60

The figures in brackets correspond to the minor conformer.

¶ $^2J_{HC^{\alpha}H}$

These values are much the same as those observed for PMRI peptides **104**, **131** and **136**, which suggests that no significant structural change occurred within this set of PMRI peptides. If PMRI hexapeptide **132** adopted a β-turn across residues -*m*Gly^2-Pro^3-Gly^4-

§ Very few cross peaks were conclusively observed for the minor conformer.

gGly^{5}-, then the values of $^{3}J_{HNC\alpha H_2}$ for Gly^{4} might be expected to reflect this, *e.g.* one large and one small value is often seen.[203] No such evidence was observed, although $\phi_4 = \pm 60°$ is accordant with, but does not rigorously establish, the presence of a β-turn (type III or III').[187,203]

So the NMR studies performed on PMRI hexapeptide **132**, in common with the other PMRI peptides described above, gave no firm indication of a preference for any rigid structure.

*(iii) CD spectra**

The CD spectra of PMRI hexapeptide **132** were recorded in four different solvent environments (water, SDS above critical micelle concentration, and TFE, with and without 57 mM sodium hydroxide)†: figure A5.

For (pseudo)peptides without aromatic residues and disulfide bonds, the CD arises from the orientation in space of the peptide bonds. In PMRI hexapeptide **132**, only the proline residue is chiral and therefore the CD is primarily due to the orientation of the prolyl tertiary amide chromophore relative to the Gly^{4} secondary amide chromophore, *i.e.* the -mGly^{2}(tertiary amide)Pro^{3}(secondary amide)Gly^{4}- fragment. The other amide bonds in the pendent PMRI peptide chains are expected to make little, if any, CD contribution.

Therefore the CD spectrum characteristic of a β-sheet structure[187,343,345,361] is not expected, even if PMRI hexapeptide **132** adopts a β-hairpin conformation under these conditions, because the β-sheet portion would contain no chiral centres and so make a negligible CD contribution. Rather, we might expect to observe a CD spectrum indicative of a β-turn.[343,361] The CD spectra recorded (figure A5) may be interpreted as follows, assuming negligible contributions from proline *cis* / *trans* isomerism (which is not usually

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* The CD spectra of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132** were recorded and interpreted by Dr. Giuliano Siligari at the EPSRC National Chiroptical Laboratory, Birkbeck College, London.

† Measurement was possible in these solvents because the sensitivity of CD spectroscopy permits use of low concentration solution [0.5 mM in this case (*i.e. ca.* 0.3 mg/mL)]. DMSO is not a suitable solvent for CD spectroscopy.

observed at such concentrations), and referring only to the -*m*Gly^2-Pro^3-Gly^4- portion. In water the structure was irregular without preferred orientations of the two amide chromophores. At supramicellar SDS concentration, the positive CD band below 195 nm is indicative of a more defined orientation between the two amide chromophores. The conformation appeared to be even more defined in TFE, but the addition of 57 mM sodium hydroxide caused substantial conformational disruption. The CD spectrum in TFE resembles that of a type II β-turn conformation (or a β-sheet, the two CD spectra are similar),[343,361] but it is not possible to conclusively state that a β-turn is adopted.

(iv) β-Turn detection by IR spectroscopy

An amide I absorption band near or below 1640 cm^{-1} in peptide or protein IR spectra (solution or solid state) may be correlated with the acceptor carbonyl of a strong i ... i+3 intramolecular hydrogen bond, such as usually exists in a β-turn.[348,362]

The Nujol mull IR spectrum of PMRI hexapeptide **132** (but not Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1**) displayed a very strong band at 1635cm^{-1}, which may, therefore, be indicative of β-turn formation. However such an absorption band was also found in the IR spectrum of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, which cannot form a β-turn. Thus the observation of the 1635cm^{-1} absorption for these PMRI hexapeptide is inconclusive.

(c) Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, 1

The quantity of pure PMRI hexapeptide **1** obtained was insufficient to permit thorough conformational investigations. The studies that were carried out are described below.

(i) Conformers

As noted in section **2.3.3***(a)(i)*, PMRI hexapeptide **1** exists in *cis* and *trans*-conformers at -*m*Gly^2-Pro^3-: its *cis* : *trans* ratio, measured by ^{1}H NMR spectroscopy, is *ca.* 30:70 (the same as that for PMRI tetrapeptide **133**). This suggests that if the desired β-hairpin type structure is adopted by PMRI hexapeptide **1**, it does not lead one conformer

to be favoured at the expense of the other [as discussed for PMRI hexapeptide **132**, see section **2.3.3***(b)(i)*].

(ii) NOESY and J *analysis*

The NOESY spectrum of Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1** was very noisy (this was not such a problem with the COSY): only an NOE between the methylene and methyl protons of the ethyl ester was detected with any certainty.‡ Consequently unambiguous assignment of the ^{1}H NMR NH resonances was not possible in this case.

The substantial overlap of the NH resonances in the ^{1}H NMR spectrum of PMRI hexapeptide **1** makes the usual *J* analysis impossible. Neither is it possible to extract *J* values from the C$^\alpha$H resonances. No attempt was made to extricate the *J* values from the COSY spectrum.

Thus no conclusions may be drawn on the conformation of Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1**, other than the existence of *cis* / *trans*-isomers at the -*m*Gly^2-Pro^3- amide bond, and the absence of any detectable, rigid, defined structure.

2.3.4 *Summary of the conformational investigations.*

NMR studies in $DMSO_{d6}$ failed to detect any defined secondary structure for PMRI peptides **120**, **104**, **122**, **131**, **136**, **132**, or **1**. In no case was evidence found for a folded structure, which was as expected for all but PMRI hexapeptides **132** and **1**. For these two PMRI hexapeptides we had hoped to detect a β-hairpin type structure, but as with the other PMRI peptides mentioned, all the NMR evidence merely pointed towards conformational averaging, *i.e.* a range of rapidly interconverting conformational states (often misleadingly called a "random coil"[340,344]). Only the proline containing PMRI peptides (**131**, **136**, **132**, and **1**) deviated from this behaviour in that they displayed slow *cis* / *trans* -*m*Gly^i-Pro^{i+1}- isomerism.

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‡ The tumbling rate of (modified) peptides of about this size is often such as to result in very weak, hence unobservable, NOEs.[340,352]

Nevertheless, no evidence against the expected structures was observed. Thus the β-hairpin type structure may be adopted / occupied by Boc-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-OEt, **132** (Xaa = Gly) and / or **1** (Xaa = Val), but if so, it is not a sufficiently rigid and / or energetically favoured arrangement in DMSO to permit its detection.

The CD spectra of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132** suggested the existence of some defined secondary structure of the -*m*Gly^2-Pro^3-Gly^4- sequence in TFE, possibly the adoption of a type II β-turn.

The combination of CD and NMR studies under suitable conditions could prove useful for future studies of the conformational behaviour of such PMRI peptides, if solubility problems can be surmounted (see section **2.5.2**).

2.4 *Conclusions.-*

2.4.1 *Synthesis and protecting group manipulations of PMRI dipeptides.*

The Goldschmidt and Wick type synthesis of PMRI dipeptides Boc-Xaaψ(NHCO)Gly-OEt, **99** (Xaa = Gly), **116(a)** (Xaa = Val), and **116(b)** (Xaa = Phe) from the corresponding Boc-amino acids [**95**, **113(a)** and **113(b)** respectively], sodium azide and monoethyl malonate, **98**, has been demonstrated to proceed in moderate yield (20-48%). Variations in the method of formation of the intermediate α-(Boc-amino)acyl azide did not improve the original procedure.

C-Terminal deprotection of these PMRI dipeptides was trivially accomplished, in near quantitative yields, by saponification.

N-Terminal deprotection using hydrogen chloride solutions was problematic: decomposition of the products occurred, as noted by Loudon and co-workers for monoacyl *gem*-diamino compounds generally [section **1.5.1***(a)(i)(5)*], yielding ammonium chloride, an aldehyde and ethyl malonamate, **118**. The extent of decomposition increased across the series **99** < **116(a)** < **116(b)**, due to stabilisation of the intermediate iminium species by the side chains. Ethyl malonamate, **118**, and ethyl malonyl-Valψ(NHCO)Gly-OEt, **120** were identified as by-products from the deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)** with ethanolic hydrogen chloride.

Satisfactory *N*-Boc deprotection was achieved using TFA, and an appropriate work-up procedure was developed. Thus, TFA.Glyψ(NHCO)Gly-OEt, **102** was obtained in 86% yield; TFA.Valψ(NHCO)Gly-OEt, **119**, in 84% yield; and TFA.Pheψ(NHCO)Gly-OEt, **124**, in 43-61% yield (decomposition is obviously still significant in this case at least).

Therefore we have demonstrated that *N*-Boc deprotection of PMRI dipeptides may be achieved quite satisfactorily, provided the side chain of the *gem*-diamino residue bearing the Boc group does not greatly stabilise the iminium intermediate on the decomposition pathway (-*g*Phe- was particularly problematic in this project).

PMRI dipeptides Fmoc-Xaaψ(NHCO)Gly, **143** (Xaa = Gly), and **144** (Xaa = Val), for use in SPS, were obtained by reprotection of the corresponding fully deprotected PMRI dipeptides, **141** and **142**, using Fmoc-OSu. Fmoc-Glyψ(NHCO)Gly, **143**, was obtained in 92% yield from Boc-Glyψ(NHCO)Gly, **103**. In contrast, Fmoc-Valψ(NHCO)Gly, **144** was obtained in only 25% yield from Boc-Valψ(NHCO)Gly, **121**, and a by-product, Fmoc-NH_2, **145** [derived from TFA.Valψ(NHCO)Gly, **142** decomposition] was also isolated in this case (in 17% yield). To the best of our knowledge, these are the first examples of *N*-Fmoc PMRI dipeptides.

2.4.2 *Elongation of the PMRI dipeptides: PMRI tripeptides and beyond.*

The syntheses of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, and Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, were used as test-beds for various peptide coupling procedures. Carbodiimide mediated couplings, in the presence of the auxiliary nucleophiles, HOSu and HOBt, gave moderate yields of the desired PMRI tetrapeptides, but profound purification difficulties resulted due to the low solubility of the products. Thus, the only satisfactory carbodiimide procedure to yield the PMRI tri- and tetrapeptides **120**, **104**, **122** and **126** used EDC / HOSu, and owed its success to the water solubility of the by-products. Indeed EDC / HOSu was the method of choice for the couplings to PMRI tri- and tetrapeptides **120**, **122** and **126**.

The method of choice for the synthesis of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** was isolation of the active ester, Boc-Glyψ(NHCO)Gly-OSu,

106 (generated using DCC), and subsequent coupling. Formation of the active ester, Boc-Valψ(NHCO)Gly-OSu, **123** using DSC, showed promise, but was not thoroughly investigated [section **2.1.2***(d)(ii)(2)*].

Carbonate mixed anhydride couplings were only successful between *gem*-diamino residues and non-malonyl residues [*e.g.* the syntheses of Boc-Xaaψ(NHCO)Gly-Pro-Gly-Xaaψ(NHCO)Gly-OEt, **132** (Xaa = Gly) and **1** (Xaa = Val)]. Attempted carbonate mixed anhydride couplings to malonyl residues merely yielded the corresponding esters, as previously observed by Goodman and co-workers and Gutman and Boltanski [see sections **1.5.1***(a)(i)(1)* and **2.1.1***(d)(iii)(3)*].

All the couplings investigated between *gem*-diamino and malonyl residues were rather slow, which is a finding general to PMRI peptide synthesis [see section **1.5.1***(a)(i)(1)*].

The moderate coupling yields generally experienced are attributed to *gem*-diamino residue decomposition during the long reactions. A further detrimental factor was difficult product isolation and purification due to the low solubility encountered in most of the elongated PMRI peptides.

Thus, judicious use of the above mentioned coupling procedures [and the deprotection procedures developed for the PMRI dipeptides, **99**, **116(a)** and **116(b)**] provided access to the initial target PMRI tetrapeptides, **104**, **122** and **126**, and the truncated target PMRI hexapeptides, **132** and **1**; but failed to furnish the ultimate target PMRI decapeptide **140**. Poor solubility of all PMRI peptides containing our target motif, and of the truncated targets, irrespective of the presence of proline or the nature of the side chains, dogged the syntheses and could only be tackled by the use of highly polar solvents; solubilisation by chemical modification was investigated to no avail.

SPS of PMRI peptides was demonstrated to be possible under somewhat modified Fmoc continuous flow conditions, but insolubility prohibited purification of the SPS product, **146**. Nonetheless the PAL handle was shown to be a suitable anchor for malonyl residues (ultimately yielding the *C*-terminal amide) and prolonged couplings (DIPCDI / HOBt) and shortened deprotections allowed incorporation of the PMRI dipepitde SPS monomers, **143**, as indicated by the Fmoc deprotection profiles (figure A4).

2.4.3 *Conformational studies.*

The aforementioned solubility problems necessitated the use of $DMSO_{d6}$ as solvent for the NMR studies. Under these conditions we were unable to detect any secondary structure formation. Thus we must conclude that our PMRI peptides do not adopt any rigid conformation(s), but rather, are in rapid equilibrium between many conformers under these conditions. This finding does not preclude the occupation of the expected β-hairpin structures (twisted or otherwise), but provides no evidence thereof.

The CD spectrum of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132** in TFE suggested some defined secondary structure of the -*m*Gly^2-Pro^3-Gly^4- sequence, possibly a type II β-turn, which would be consistent with a β-hairpin structure.

Weak evidence suggesting a β-sheet forming tendency of our structural motif is provided by the low solubility of the PMRI peptides that featured it, since β-structure formation by blocked peptides and intermolecular association, leading to insolubility are causally linked. The fact that solvent systems used to disrupt peptide β-structure [*i.e.* DMSO, TFE, lithium chloride / THF, *etc*: see section **2.1.4***(a)*] were effective against the insolubility of PMRI peptides in this study further supports the view that our PMRI peptides exhibit a β-structure forming tendency. However there is no suggestion of well-defined secondary structure formation by our PMRI peptides.

It is possible that the insolubility observed for our PMRI peptides (tetra- and above) was merely due to unfortunate sequence choice, rather than an inherent property of the target motif, for the PMRI peptides were designed to include side chains with a high β-sheet forming propensity. Only further investigation with alternative side chains can shed light upon this. However, comparison with blocked natural peptides of analogous sequence indicates that, in the glycine case, the onset of aggregation / insolubility / β-sheet formation does not occur until at least the pentapeptide.[363-365]

In summary, we have developed synthetic methodology that permits access to PMRI peptides containing the structural motif $[Xaa\psi(NHCO)Gly]_n$, $n \geq 2$, using solution or SPS. This motif was expected to be predisposed towards β-sheet formation but we have been unable to produce evidence to that effect, due to difficulties caused by low

solubility (which may itself be weak evidence thereof). Thus we are unable to support or refute the conflicting conformational predictions of Dauber-Osguthorpe and co-workers and Aléman, Puiggalí and co-workers, based upon molecular modelling [section **1.5.3***(b)*], nor add substantially to the body of experimental conformational studies of PMRI peptides.

2.5 *Future Work.-*

2.5.1 *Synthetic methodology.*

As mentioned above, couplings between *gem*-diamino and malonyl residues are slow, a factor that contributes to their inefficiency. Hence further investigation of alternative coupling procedures is warranted.

(a) New coupling reagents

In view of Verdini and co-workers' successful anchorage of a malonyl residue to a solid support using carbonyl bis(*N*-methylimidazolium) dichloride, **89** [section **1.5.1***(b)(iii)*], this reagent may also prove useful for solution phase synthesis. The application of the related coupling reagent, carbonyldiimidazole (and other variants thereof)[100] is also worthy of investigation, for it is yet to find widespread use in PMRI peptide synthesis.

The literature abounds with new peptide coupling reagents, the popularity of which waxes and wanes with their (low-cost) commercial availability. Two recent examples that may prove useful in the PMRI peptide context are bis{[4-(2,2-dimethyl-1,3-dioxolyl)]methyl}carbodiimide, which gives rise to a urea that is readily removed with water,[366] and the amidation catalyst 3,4,5-trifluorobenzeneboronic acid.[367]

(b) Non-carbonate mixed anhydrides

Following Goodman and co-workers' report of failed mixed anhydride couplings [see section **1.5.1***(a)(i)(1)*] there has been little evaluation of their utility in PMRI peptide synthesis. However, in the light of the mechanism of malonyl residue esterification during attempted carbonate mixed anhydride couplings [see section **2.1.1***(d)(iii)(3)*], a re-investigation thereof is justified. The use of a non-carbonate mixed anhydride would

preclude undesired ester formation (although generation of the proposed ketene intermediate is still possible) and may thus enable the desired coupling, either with an amine [*c.f.* section **2.1.1***(d)(ii)*] or an auxiliary nucleophile, generating an active ester [*c.f.* section **2.1.1***(d)(iii)(3)*]. Appropriate reagents are pivaloyl chloride or *iso*-propenyl chloroformate (although this yields a carbonate mixed anhydride, subsequent elimination or nucleophilic displacement generates acetone, not an alkoxide or alcohol, so is unlikely to lead to ester formation).[368]

The influence of the C^2 allyl protecting group [see section **1.5.1***(a)(ii)(1)*] upon the course of attempted mixed anhydride coupling to malonyl residues is, as yet, untested.

(c) Active esters

Further examination of malonyl residue active ester formation using DSC, following up that described herein [section **2.1.2***(d)(ii)(2)*], is merited.

(d) SPS

Although our synthesis of Fmoc-Glyψ(NHCO)Gly, **143** was successful, the introduction of Fmoc leading to Fmoc-Valψ(NHCO)Gly, **144**, was unsatisfactory. Other reagents, such as Fmoc-Cl and Fmoc-N_3 may improve this synthesis.

Use of an alternative resin (*e.g.* the Kaiser oxime resin, with which anchorage may be more difficult) would permit the SPS of a PMRI peptide blocked at the *C*-terminus with a group other than a primary amide.[294] Following the rationale of Kemp,[216] such a reduction in the number of hydrogen bond donors not satisfied by intermolecular hydrogen bonding could result in less aggregation and thus facilitate purification and further study of PMRI peptides analogous to **146**.

(e) Solvents

In this project, couplings leading to and involving PMRI peptides of low solubility were generally carried out in DMF. In our investigation of solubilisation [section **2.1.4**] we found that DMSO, TFE, HFIP and lithium chloride / THF dissolved Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**. Therefore couplings in these solvents (during solution or SPS) could prove efficient, although the isolation and purification

problems we encountered would not be alleviated. TFE and / or HFIP have been employed with natural peptides, diluted with dichloromethane [see section **2.1.4***(a)(i)*]: such a solvent system may also be useful in PMRI peptide synthesis. However, with these fluoroalcohols there is the possibility of ester formation and thus rather slow or incomplete reactions [see section **2.1.4***(a)(i)*].

2.5.2 *The target motif: [Xaaψ(NHCO)Yaa]$_n$, $n \geq 2$.*

Although the results of Gardner and Gellman demonstrate that our target motif may not lead to β-structure any more stable than that of the corresponding natural peptides [see section **1.5.3***(b)(iv)(2)*], there is still the question of what secondary structure, if any, such PMRI peptides favour. Furthermore, the motif remains an interesting category of structure [*c.f.* section **1.5.3***(b)(iv)(1)*], worthy of additional experimental investigation. Thus, various avenues for redesign are outlined below, with the caveat that too much constraint may lead to secondary structure formation influenced more by the constraints than any inherent tendency of the motif.

(a) Alternative residues

Alternative choices of Xaa and Yaa permit solubility to be designed into our target motif. Attempts, in this study, to thereby effect solubility in organic solvents failed. But conversely, Xaa = Lys could result in aqueous solubility.

(b) Templates

Mutter's "template-assembled synthetic protein" concept,[369] and Nowick and co-workers' oligourea "molecular scaffold"[370] permit secondary structure formation by peptide chains attached to a "template" or "scaffold". Thus, PMRI peptide chains, featuring our target motif, could be attached to such a template with the potential result that intramolecular β-sheet formation could occur without intermolecular aggregation.

(c) Alternative turns

Rather than the -Pro-Gly- sequence employed in this study, another turn forming unit could be employed. Use of a less flexible β-turn mimic, *e.g.* those of Kelly and co-

workers,[371] Feigel,[372] or Kemp and Li,[373,374] could favour β-hairpin formation between the pendant PMRI peptide chains.

(d) Extra constraints

More forcing constraints could be applied to the target motif, *e.g.* cyclisation using a second (natural or peptidomimetic) turn unit,[375] or by the incorporation of cystine.[347] Alternatively, those amide hydrogen bond donors not involved in the desired β-hairpin formation [*i.e.* those that point outwards in figure 14(a)] could be blocked (*e.g.* by methylation). This course of action would serve the dual purpose of disfavouring undesired conformations stabilised by hydrogen bonds involving those donors, and diminishing intermolecular hydrogen bonding and thus aggregation. However, further conformational flexibility could result due to the ready *cis* / *trans* isomerisation of tertiary amides.

Chapter 3: Experimental

3.1 *General Methods, Equipment, and Materials.-*

3.1.1 *General Methods.*

Glassware used for moisture sensitive reactions was dried by washing with acetone before heating in an oven at *ca.* 120°C overnight, then allowed to cool in a desiccator over calcium chloride / silica gel.

Reactions requiring an inert atmosphere were performed under nitrogen or argon; those in a dry atmosphere were initially flushed with nitrogen or argon and kept dry using a calcium chloride / silica gel guard tube.

Reactions carried out at -10 or -15°C were cooled using an ice-salt bath; the temperature refers to that of the bath.

Solvents were removed using a rotary evaporator with a water condenser or dry-ice / acetone cold finger and under water or oil pump vacuum, as required.

3.1.2 *Solvents.*

Ether, which refers to diethyl ether, and THF were pre-dried over sodium wire and then distilled from sodium / benzophenone ketyl in an inert atmosphere, prior to use.

Toluene was pre-dried over sodium wire and then distilled from sodium under an inert atmosphere, prior to use.

Chloroform was washed with water, dried over calcium chloride, distilled from calcium chloride, and stored in dark bottles in an inert atmosphere.

Petrol, which refers to petroleum b.p. 60-80°C, was distilled; as were acetone and ethyl acetate.

Dichloromethane was distilled from phosphorus pentoxide.

Ethanol was dried by the Lund and Bjerrum method.[376]

DMF was fractionally distilled under water pump vacuum, with a nitrogen bleed; the initial wet fore-run was discarded and dry DMF collected at *ca.* 50°C and stored over activated 4 Å molecular sieves, in dark bottles, in an inert atmosphere.

Acetonitrile was distilled from calcium hydride.

All other solvents were used as supplied.

Solvent A refers to water containing 0.1% TFA. Solvent B refers to commercial acetonitrile / water (90:10) containing 0.1% TFA.

3.1.3 *Reagents.*

Amberlite IR 120(+) ion-exchange resin was soaked overnight in 2 M hydrochloric acid, the resin filtered off and washed with water until the washings were neutral.

Ethyl chloroformate was fractionally distilled at atmospheric pressure, collecting the fraction with b.p. 91-92°C.

HCl.Gly-OEt was crystallised from ethanol.

5 to 6 M HCl in ethanol was prepared by bubbling HCl through ethanol to produce a saturated solution. An aliquot of this solution was titrated against 2 M sodium hydroxide (phenolphthalein indicator) and the remainder diluted with the determined volume of ethanol to produce a 5 to 6 M solution (precise molarity as stated in procedures).

NMM and pyridine were distilled from calcium hydride.

Triethylamine was distilled from lithium aluminium hydride.

Lithium chloride was dried at 150°C, over phosphorus pentoxide, under vacuum for 5 h.

All solutions mentioned were aq. unless otherwise stated. Brine refers to a sat. solution of sodium chloride.

3.1.4 *Chromatography.*

TLC on Whatman aluminium backed UV_{254} silica gel plates, was routinely used as a qualitative guide to the progress of reactions, and the purity of compounds. The following solvent systems were used for TLC: mixture A [ethyl acetate / petrol (70:30)], mixture B [ethyl acetate / petrol (60:40)], mixture C [ethyl acetate / petrol (50:50)], mixture D [chloroform / methanol / acetic acid (80:18:2)], mixture E [chloroform / methanol / acetic acid (90:8:2)], and mixture F [chloroform / methanol / acetic acid

(95:3:2)]. The chromatograms were viewed under UV light and visualised using a ninhydrin dip solution (0.2% ninhydrin in absolute ethanol) followed by heating.

Flash column chromatography was carried out under medium pressure on Amicon 60Å 35-70 mesh silica gel, following the guidelines of Still *et. al.*[377] The columns were packed with a slurry of silica in the initial eluent. The substrates were applied to the columns either preabsorbed onto silica from a suitable solvent, or as a solution or slurry in the initial eluent, as noted. Moderate pressure was applied to the columns using hand bellows. When acetic acid was used as a component of the eluent, toluene was added to the pooled fractions in order to aid the evaporation of the acetic acid by azeotrope formation; some samples retained toluene, in those cases the toluene was removed by washing with petrol.

Analytical HPLC was performed on a LKB Bromma twin-pump HPLC instrument, with a Vydak C_{18} wide pore reverse phase column, a high-pressure gradient manager and a diode-array detector, using a gradient of solvent B in solvent A.

3.1.5 *Analysis and Spectroscopy.*

Melting points were recorded on an Electrothermal apparatus and are uncorrected.

Elemental analyses were recorded on a Carlo Erba 1106 Elemental Analyser.

Optical rotations were recorded on a Perkin-Elmer 141 Polarimeter, with $[\alpha]_D$ values quoted in 10^{-1} deg cm^2 g^{-1}, and concentration (*c*) quoted in g / 100 mL.

IR spectra (Nujol mull, sodium chloride plates, unless otherwise stated) were recorded on a Perkin Elmer 1600 Series FTIR instrument, or a Perkin-Elmer 1310 Infrared Spectrophotometer.

UV spectra were recorded on a Perkin-Elmer Lambda 3 UV/VIS Spectrophotometer.

^1H NMR spectra were recorded at 270 MHz and ^{13}C NMR spectra at 67.8 MHz on a Jeol JNM-GX270 instrument, or at 400 MHz and 100.4 MHz on a Jeol-EX400 instrument, as indicated. All spectra were recorded at room temperature (*ca.* 21°C), unless otherwise stated. Chemical shifts (δ) are quoted in ppm with TMS as standard [spectra were referenced to internal TMS or to solvent ($DMSO_{d6}$ at 2.50 ppm and 39.6

ppm, and chloroform at 7.26 ppm and 77.0 ppm, for ^{1}H and ^{13}C spectra respectively) as noted]. Coupling constants (J) are given in Hz. The assignment of ^{13}C NMR spectra was assisted by DEPT experiments. The assignment of ^{1}H spectra was assisted by D_2O exchange experiments where appropriate. It should be noted that, for all ^{1}H NMR spectra run in $DMSO_{d6}$ there is a broad water peak at *ca.* 3.3 ppm which somewhat obscures other resonances in that vicinity. Suppression of this signal was not carried out in order to avoid saturation transfer to exchangeable protons, suppression of signals of interest, and / or distortion of the spectra.[241] With some samples, immediate accumulation after the addition of D_2O enabled the obscured resonances to be observed. All integrals are quoted to the nearest integer, except when two (or more) species are present: in such cases well resolved signals due to each species are integrated to one decimal place. All variable temperature experiments and 2D spectra were recorded on the Jeol-EX400 instrument. Variable temperature experiments were performed by heating the sample with a stream of dry air, the data was accumulated once the sample was held at the desired temperature for *ca.* 3 m and the temperature was stable to within ± 0.6°C. Plots of δ_{NH} against temperature were produced using CA - Cricket Graph III (Macintosh version 1.0) and gradients measured using linear curve fitting.

Mass spectra were recorded on a VG Autospec, a VG Analytical 7070E, a VG Masslab Model 12-253 quadrupole, or a Kratos MS80 instrument. All mass spectra presented are positive FAB spectra, with NBA used as the matrix, unless otherwise stated. Electron impact (E.I.) spectra were recorded with an ionising potential of 70 eV. Chemical ionisation (C.I.) spectra were recorded using *iso*-butane as the reagent gas. The electrospray spectrum was recorded at 2 kV in methanol / water / acetic acid (49.9:49.9:0.2) and has an expected calibration error of at least 0.4 Da. In the mass spectra of salts, M^+ refers to the cation of the salt concerned. Negative ion (FAB) mass spectra are presented only for salts. The work of De Angelis *et al.* proved helpful in the assignment of PMRI peptide mass spectrometric fragments.[179,180]

CD spectra were recorded with a nitrogen flushed JASCO spectropolarimeter J720, using 4 s time constant, 10 nm / minute scan speed, spectral bandwidth of 1 nm and 0.02 cm pathlength cell. The spectra are reported as $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ ($M^{-1}cm^{-1}$).

3.2 *Experimental Procedures.-*

3.2.1 *General chemicals.*

(a) tert-*Butoxycarbonyl protection of glycine*[242,378]

A solution of glycine (10.0 g, 0.13 mol) in 1,4-dioxane / water (2:1, 400 mL) was cooled to 0°C and 1 M sodium hydroxide (133 mL, 0.13 mol) added with stirring. Di-*tert*-butyl dicarbonate (31.9 g, 0.15 mol) was added and the solution stirred at 0°C for 5 m. After stirring for a further 30 m at r.t. the solution was concentrated *in vacuo* to *ca.* 250 mL. The resultant aq. solution was cooled to 0°C and an equal volume of ethyl acetate added. The mixture was stirred and acidified to pH 2 / 3 with 1 M potassium hydrogen sulfate. The mixture was extracted twice with ethyl acetate (2 x 150 mL), the extracts combined, dried over sodium sulfate and evaporated to produce a colourless solid. Recrystallisation from ethyl acetate / hexane yielded Boc-glycine, **95**, as a colourless crystalline solid (13.1 g, 75 mmol, 56%), m.p. 85-88°C (lit.: 86.5-87.5°C[242], 93-94°C[379], 85-89°C[380]) (Found: C, 48.5; H, 7.2; N, 7.65. Calculated for $C_7H_{13}NO_4$: C, 48.0; H, 7.5; N, 8.0%); ν_{max}/cm^{-1} 3405m (NH), 3340m (NH), 3100br,s (OH), 1750vs (carboxylic acid), 1670vs (Boc carbonyl), 1535vs (amide II), 1410vs, 1335w, 1300m, 1280s, 1255m, 1215vs, 1200vs, 1165vs, 1055m, 1030w, 960s, 885w and 860s; δ_H(270 MHz; $DMSO_{d6}$) 1.34 (0.7 H, s, Boc minor conformer), 1.37 (8.3 H, s, Boc major conformer), 3.52 (0.3 H, br d, *J* 7.5, $C^{\alpha}H_2$ minor conformer), 3.56 (1.7 H, d, *J* 6.2, $C^{\alpha}H_2$ major conformer), 6.68 (0.1 H, br t, NH minor conformer), 7.06 (0.9 H, t, *J* 6.2, NH major conformer) and 12.50 (br s, CO_2H); δ_H(270 MHz; $CDCl_3$; TMS) 1.46 (9 H, s, Boc), 3.90 (0.7 H, br d, *J* 4.5, $C^{\alpha}H_2$ minor conformer), 3.97 (1.3 H, d, *J* 5.7, $C^{\alpha}H_2$ major conformer), 5.17 (0.6 H, br s, NH major conformer), 6.83 (0.4 H, br s, NH minor conformer) and 9.55 (1 H, br s, CO_2H) [lit.,[381] (90 MHz; $CDCl_3$; TMS; -30°C) 1.47 (Boc), 3.95 ($C^{\alpha}H_2$), 5.15 (0.1 H, t, *J* 5.5, NH), 7.68 (0.9 H, t, *J* 4.1, NH) and 12.5 (CO_2H)]; m/z (C.I.) 176 [$(M+H)^+$, 12%], 120 [100, (M+H) - CH_2CMe_2], 102 (13, 120 - H_2O) and 76 (26, 120 - CO_2).

(b) Coupling of Boc-Pro and HCl.Gly-OEt

A solution of HCl.Gly-OEt (2.9 g, 21 mmol) and Boc-Pro (4.5 g, 21 mmol) in THF (50 mL) was cooled to -10°C. Triethylamine (2.9 mL, 2.1 g, 21 mmol), HOBt

hydrate (3.2 g, 21 mmol) and DCC (4.4 g, 21 mmol) were successively added at -10°C, with stirring. The mixture was stirred in a dry atmosphere, at -10°C for 4 h, and then at r.t. for a further 5 days. The colourless precipitate was filtered off and washed with ethyl acetate (100 mL). The filtrate was washed successively with 5% citric acid (100 mL), 5% sodium hydrogen carbonate (100 mL) and water (100 mL plus 10 mL brine) and separated. Each aq. layer was back extracted with ethyl acetate (3 x 33 mL) and the combined organic extracts dried over sodium sulfate, evaporated, and dried under high vacuum. Column chromatography of the resultant yellow oil (preabsorbed onto silica from ethyl acetate solution) with ethyl acetate / petrol (70:30) as eluent yielded Boc-Pro-Gly-OEt as a colourless gum which retained solvent [4.7 g, *ca.* 75% (prior to analysis the gum was subjected to a few dissolution / evaporation cycles with deuterochloroform as solvent, in order to ensure that deuterochloroform was the only solvent retained)], (Found: C, 54.1; H, 7.85; N, 9.15. Calculated for $C_{14}H_{24}N_2O_5.0.1CDCl_3$: C, 54.2; H, 7.8; N, 8.95%); $[\alpha]_D^{16}$ - 58.1 (*c* 1.98 in ethanol*) [lit.,[382] -58.0 (*c* 7.8 in ethanol)]; ν_{max} /cm^{-1} (neat) 3530br,w (NH), 3310br,m (NH), 3085w (NH), 2980s (CH), 2955m (CH), 2885m (CH), 1755s (ester carbonyl), 1695vs (Boc carbonyl), 1665br,vs (amide carbonyl), 1535br,s (amide II), 1470m (CH), 1455m (CH), 1395br,vs, 1365s, 1250s, 1195vs, 1165vs, 1125s, 1095m, 1025m, 975w, 920w, 890w, 850w, 775w and 730m; δ_H(270 MHz; $CDCl_3$; TMS) 1.28 (3 H, t, *J* 7.1, OCH_2CH_3), 1.47 (9 H, s, Boc), 1.83-2.40 (4 H, br m, Pro$C^{\beta}H_2$ and $C^{\gamma}H_2$)†, 3.29-3.57 (2 H, br m, Pro$C^{\delta}H_2$)‡, 4.00 (1 H, br dd, *J* 6.0 and 18.0, Gly$C^{\alpha}H_2$)§, 4.06 (1 H, dd, *J* 6.0 and 18.0, Gly$C^{\alpha}H_2$), 4.21 (2 H, q, *J* 7.1, OCH_2CH_3), 4.33 (1 H br s, Pro$C^{\alpha}H$)§, 6.60 (0.5 H, br s, NH)¶ and 7.28 (0.5 H, br s, NH)¶; δ_C(100.4

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* Concentration corrected for deuterchloroform content of 9 mole %, as indicated by elemental analysis.

† On warming to 50°C, there was only a small change in the appearance of this multiplet.

‡ At r.t this signal consisted of two overlapping broad singlets: major, δ_H 3.47 and minor δ_H 3.37. At 50°C: δ_H 3.45 (br s).

§ Sharpened at 50°C.

¶ At 50°C these two signals virtually coalesced: δ_H 6.86.

MHz; $CDCl_3$) 14.1 (OCH_2CH_3), 23.6 (br, Pro$C^{\gamma}H_2$ minor conformer), 24.4 (br, Pro$C^{\gamma}H_2$ major conformer), 28.3 [(CH_3)$_3$C], 30.9 (br, Pro$C^{\beta}H_2$), 41.2 (Gly$C^{\alpha}H_2$), 47.0 (Pro$C^{\delta}H_2$), 59.9 (br, Pro$C^{\alpha}H$), 61.3 (br, OCH_2CH_3), 80.5 [(CH_3)$_3$C], 154.6 (br, Boc CO minor conformer), 155.7 (br, Boc CO major conformer), 169.6 (GlyCO), 172.3 (br, ProCO major conformer) and 172.9 (br, ProCO minor conformer); m/z (E.I.)* 300 (M^+, 300.1706, $C_{14}H_{24}N_2O_5$ requires 300.1685, 1%), 244 (3, M - CH_2CMe_2), 227 (3, 244 - OH), 199 (7, 244 - CO_2H), 170 {19, Boc$\overline{N[CH_2]_3CH}^+$}, 114 (79, 170 - CH_2CMe_2), 70 {100, $H\overline{N[CH_2]_3CH}^+$}, 57 (62, Bu^{t+}), 41 (14) and 30 (10).

(c) N-*Terminal deprotection of Boc-Pro-Gly-OEt with ethanolic hydrogen chloride*

Boc-Pro-Gly-OEt (2.3 g, *ca.* 7.5 mmol) was dissolved in 6 M HCl in ethanol (50 mL) and stirred at r.t., in a dry atmosphere for 2 h. The solution was evaporated to dryness to provide a yellow oil. The oil was dissolved in water and freeze dried to yield HCl.Pro-Gly-OEt, **129** (1.7 g, 95%), as a hygroscopic, glassy, yellow syrup; $[\alpha]_D^{18}$ - 41.2 (*c* 0.61 in ethanol); ν_{max} /cm^{-1} 3650-2400br,m (NH_2^+ and H_2O), 3325br,m (NH), 3210br,m (NH), 2725m (NH_2^+), 1740s (ester carbonyl), 1680vs (amide carbonyl), 1555br,m (amide II and NH_2^+), 1300m, 1210vs, 1115w, 1025m, 975w, 940w, 865w and 720m; δ_H(270 MHz; $DMSO_{d6}$) 1.19 (3 H, t, *J* 7.0, OCH_2CH_3), 1.79-1.96 (3 H, m, Pro$C^{\beta}H_2$ and $C^{\gamma}H_2$), 2.23-2.40 (1 H, m, Pro$C^{\beta}H_2$ and $C^{\gamma}H_2$), 3.19 (2 H, br s, Pro$C^{\delta}H_2$), 3.77-4.09 (2 H, m, Gly$C^{\alpha}H_2$), 4.10 (2 H, q, *J* 7.1, OCH_2CH_3), 4.23 (1 H br s, Pro$C^{\alpha}H$), 8.56 (1 H, br s, ProNH_2^+)†, 8.99 (0.2 H, t, *J* 5.3, GlyNH minor conformer), 9.13 (0.8 H, t, *J* 5.9, GlyNH major conformer) and 10.23 (1 H, br s, ProNH_2^+)†; δ_C(100.4 MHz; $DMSO_{d6}$) 14.2 (OCH_2CH_3), 23.6 (Pro$C^{\gamma}H_2$), 29.8 (Pro$C^{\beta}H_2$), 40.9 (Gly$C^{\alpha}H_2$), 45.6 (Pro$C^{\delta}H_2$), 58.7 (Pro$C^{\alpha}H$), 60.7 (OCH_2CH_3), 168.7 (GlyCO minor conformer), 168.9 (GlyCO major conformer), 169.3 (ProCO major conformer) and 170.6 (ProCO minor conformer); m/z 401 [(2M-H)$^+$, 14%], 201 (100, M^+) and 173 (10, M - C_2H_4); m/z (-ve) 275 [(M+2^{37}Cl)$^-$, 3%], 273 [21, (M+^{37}Cl+^{35}Cl)$^-$], 271 [33, (M+2^{35}Cl)$^-$], 245 (6, 273 -

* The mass spectrum also contained the following peaks, due to deuterochloroform: 88 (3, $DC^{37}Cl_2^+$), 86 (19, $DC^{35}Cl^{37}Cl^+$) and 84 (29, $DC^{35}Cl_2^+$).

† At 80°C these two signals coalesced: δ_H 9.62.

C_2H_4), 243 (13, 271 - C_2H_4), 237 [7, $(M-H+^{37}Cl)^-$], 235 [27, $(M-H+^{35}Cl)^-$], 209 (10, 237 - C_2H_4), 207 (30, 235 - C_2H_4), 190 [34, $(^{37}Cl+NBA)^-$], 188 [100, $(^{35}Cl+NBA)^-$] and 171 [21, (M-2H) - C_2H_4].

(d) Hydrogenolysis of benzyl ethyl malonate

A solution of benzyl ethyl malonate (Aldrich, 85% tech.‡, 13.8 g, 62 mmol) in absolute ethanol (250 mL) was hydrogenolysed over 10% palladium on carbon (1.4 g), under atmospheric pressure, at r.t. for 5 h. The solution was filtered through Celite, and the ethanol removed by evaporation. Fractional distillation of the residual oil under vacuum yielded monoethyl malonate, **98**, (5.3 g, 65%), b.p. 89°C, 0.2 mmHg (lit.,[383] 77-78°C, 0.15 mmHg) (Found: C, 44.9; H, 6.35. Calculated for $C_5H_8O_4$: C, 45.45; H, 6.1%); ν_{max} (liquid film)/cm^{-1} 3500-2500br,m (CO_2H), 2985s (CH), 1730br,vs (carbonyls), 1395m, 1375s, 1325vs, 1205s, 1160vs and 1030s (C-O); δ_H(400 MHz, $CDCl_3$, TMS) 1.30 (3 H, t, *J* 7.1, CH_2CH_3), 3.45 (2 H, s, $COCH_2CO$), 4.24 (2 H, q, *J* 7.2, CH_2CH_3) and 10.45 (1 H, br s, CO_2H) [lit.,[384] 1.27 (3 H, t, *J* 7, CH_2CH_3), 3.40 (2 H, s, $COCH_2CO$), 4.22 (2 H, q, *J* 7, CH_2CH_3) and 8.63 (1 H, s, CO_2H)]; m/z (E.I.) 133 [$(M+H)^+$, 6%], 115 [10, (M+H) - H_2O], 105 [12, (M+H) - CH_2CH_2], 87 (23, 105 -H_2O), 70 (9), 61 (15), 43 (100, CH_2COH^+) and 29 (28, CHO^+).

3.2.2 *Gly series.*

(a) Preparation of Boc-Glyψ(NHCO)Gly-OEt, ***99***

(i) Original procedure

Boc-glycine, **95** (5.9 g, 33 mmol) was dissolved in THF (67 mL) and cooled to 0°C in an inert atmosphere. Tri-*n*-butyl amine (6.2 g, 8.0 mL, 34 mmol) followed by ethyl chloroformate (3.7 g, 3.3 mL, 34 mmol) were added slowly, with stirring and the temperature maintained at 0°C. Stirring was continued at 0°C for 30 m. An ice cold

‡ Use of benzyl ethyl malonate supplied by Fluka (">97% HPLC"), yielded crude monoethyl malonate, **98**, which required two distillations to obtain pure monoethyl malonate, **98**, in a consistantly lower yield (50%) than that obtained from the Aldrich benzyl ethyl malonate.

solution of sodium azide§ (4.4 g, 67 mmol) in the minimum volume of water (20 mL) was added and stirring continued for 1 h at 0°C. Ice cold portions of ethyl acetate (335 mL) and sat. sodium hydrogen carbonate (335 mL) were added and the mixture shaken and separated. The organic portion was washed with ice cold brine (335 mL), dried over magnesium sulfate and evaporated at <25°C.¶ The resultant yellow oil was dissolved in toluene (67 mL) and heated to 70°C in an inert atmosphere, with stirring, for *ca.* 1 h (during which vigorous effervescence occurred), until IR analysis indicated all the acyl azide, **96**, [ν_{max}(tol) 2140 cm^{-1}] had converted to the isocyanate, **97**, [ν_{max}(tol) 2245 cm^{-1}]. The solution was maintained at 70°C and monoethyl malonate, **98** (5.3 g, 40 mmol) was added. Stirring was continued at 70°C for *ca.* 45 m (during which further effervescence occurred), until IR analysis indicated all the isocyanate, **97**, was consumed; after which the solution was cooled to r.t. and evaporated. The resultant yellow residue was redissolved in the minimum volume of toluene (30 mL) and petrol (100 mL) added. The resultant precipitate was filtered off, washed with toluene / petrol (2:1) and dried under vacuum. Further crude Boc-Glyψ(NHCO)Gly-OEt, **99**, was obtained from the filtrate by addition of ethyl acetate (50 mL) and successive washes with 5% sodium hydrogen carbonate (50 mL), 5% citric acid (50 mL) and water (50 mL). The organic portion was dried over sodium sulfate, evaporated and dried under high vacuum. Column chromatography (sample preabsorbed from ethyl acetate solution) with ethyl acetate / petrol (70:30) as eluent, followed by recrystallisation from toluene / petrol yielded Boc-Glyψ(NHCO)Gly-OEt, **99** (2.2 g, 25%)*, as colourless needles, m.p. 86-87°C (Found: C,

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§ This reaction has been performed safely using up to 10 g of sodium azide.

¶ When the flask was removed from the rotary evaporator, air was readmitted *via* a calcium chloride / silica gel drying tube.

* The column chromatography also yielded crude *N*,*N'*-bis(*N*-Boc-aminomethyl)-urea, **100**, as a colourless solid. This material was very difficult to purify, but a relatively pure sample was obtained by exhaustive washing with ethyl acetate; δ_H (270 MHz; $DMSO_{d6}$) 1.37 (9 H, s, Boc), 4.21 (2 H, t, *J* 6.0, NHCH_2NH), 6.58 (1 H, br m, BocNH) and 7.35 (1 H, br m, NH); m/z (C.I.) 391 (2%), 319 [3, $(M+H)^+$], 263 [10, (M+H) - CH_2CMe_2], 207 [20, (M+H) - CH_2CMe_2], 202 (20, 263 - H_2O, CO, NH), 183 (11), 146 (100, 202 -

50.7; H, 7.8; N, 10.75. $C_{11}H_{20}N_2O_5$ requires C, 50.75; H, 7.75; N, 10.75%); ν_{max} /cm^{-1} 3345vs (NH), 1745vs (ester carbonyl), 1690vs (Boc carbonyl), 1665vs (amide carbonyl), 1545s (amide II), 1525vs (amide II), 1415w, 1390m, 1340m, 1320s, 1280vs, 1255s, 1225m, 1170m, 1150vs, 1120s, 1045m, 965w, 945w and 900m; δ_H(270 MHz; $CDCl_3$; TMS) 1.29 (3 H, t, *J* 7.1, OCH_2CH_3), 1.44 (9 H, s, Boc), 3.31 (2 H, s, $COCH_2CO$), 4.20 (2 H, q, *J* 7.2, OCH_2CH_3), 4.55 (2 H t, *J* 6.4, $HNCH_2NH$), 5.70 (1 H, br s, BocNH) and 7.84 (1 H, br s, NH); δ_C(67.8 MHz; $CDCl_3$) 13.9 (OCH_2CH_3), 28.2 [$(CH_3)_3C$], 41.6 ($COCH_2CO$), 46.0 ($HNCH_2NH$), 61.5 (OCH_2CH_3), 80.0 [$(CH_3)_3C$], 155.9 (Boc CO), 166.4 (CO) and 168.2 (CO); m/z 261 [$(M+H)^+$, 35%], 205 [100, (M+H) - CH_2CMe_2], 144 (39, 205 - H_2O, CO, NH) and 132 [31, (M+H) - $BocNCH_2$].

(ii) Modified procedure

Boc-glycine **95** (13.5 g, 77 mmol) was dissolved in THF (150 mL) and cooled to 0°C in an inert atmosphere. Tri-*n*-butyl amine (14.3 g, 18.3 mL, 77 mmol) followed by ethyl chloroformate (8.5 g, 7.5 mL, 78 mmol) were added slowly, with stirring and the temperature maintained at 0°C. Stirring was continued at 0°C for 30 m. An ice cold solution of sodium azide (10.0 g, 154 mmol) in the minimum volume of water (37 mL) was added and stirring continued for 1 h at 0°C. Ice cold portions of ethyl acetate (750 mL) and sat. sodium hydrogen carbonate (750 mL) were added and the mixture shaken and separated. The organic portion was washed with ice cold brine (750 mL), dried over magnesium sulfate and evaporated at <25°C.† The resultant yellow oil was dissolved in toluene (150 mL) and heated to 70°C in an inert atmosphere, with stirring, for *ca.* 1 h (during which vigorous effervescence occurred), until IR analysis indicated all the acyl azide, **96**, [ν_{max}(tol) 2140 cm^{-1}] had converted to the isocyanate, **97**, [ν_{max}(tol) 2250 cm^{-1}]. The solution was maintained at 70°C and monoethyl malonate, **98** (10.2 g, 77 mmol) added. Stirring was continued at 70°C for *ca.* 5 m (during which further effervescence

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CH_2CMe_2), 134 (47), 130 (31, $H_2CNHBoc^+$), 118 (28), 102 (24), 85 (23, 146 - H_2O, CO, NH), 74 (34), 73 (57, $H_2NCONHCH_2^+$), 62 (59) and 61 (48).

† When the flask was removed from the rotary evaporator, air was readmitted *via* a calcium chloride / silica gel drying tube.

occurred), until IR analysis indicated all the isocyanate, **97**, was consumed. The solution was cooled to r.t., diluted with ethyl acetate (750 mL) and washed with water (750 mL). The organic portion was dried over sodium sulfate and evaporated. Column chromatography (sample preabsorbed from ethyl acetate solution) with ethyl acetate / petrol (70:30) as eluent, followed by recrystallisation from toluene / petrol yielded Boc-Glyψ(NHCO)Gly-OEt, **99** (8.4 g, 42%), as colourless needle crystals, identical to the above.

*(b) Reaction of Boc-Glyψ(NHCO)Gly-OEt, **99**, with* tert-*Butyldimethylsilyl trifluoromethanesulfonate*[320,321]

Boc-Glyψ(NHCO)Gly-OEt, **99** (105 mg, 0.40 mmol) was dissolved in dichloromethane (1.5 mL) and the solution cooled to 0°C in an inert atmosphere. Triethylamine (56 μL, 41 mg. 0.40 mmol) and *tert*-butyldimethylsilyl trifluoromethanesulfonate (93 μL, 107 mg, 0.40 mmol) were successively added, and stirring continued at 0°C for 30 m. After this time TLC (mixture A) indicated the formation of a new compound (R_F 0.7), the presence of residual starting material, **99** (R_F 0.4), and a base-line spot. The reaction mixture was allowed to warm to r.t. and stirring continued. After a further 90 m, TLC indicated the formation of a second new compound (R_F 0.5) in addition to that formed initially, residual starting material, **99**, and the base-line spot. The reaction mixture was diluted with dichloromethane (13.5 mL) and washed with ice-cold sat. sodium hydrogen carbonate (10 mL). The organic layer was dried over sodium sulfate and evaporated. The resultant residue was taken up in ether and filtered (to remove triethylammonium trifluoromethanesulfonate)[321]. The filtrate was evaporated to dryness. Column chromatography of the residue (applied as a solution in the initial eluent), with ethyl acetate / petrol / triethylamine (49:49:2 then 98:0:2) as eluent, yielded: a colourless oil [24 mg, 16% (assuming mono silylated), R_F 0.7 (mixture A)] which decomposed prior to analysis {^{1}H NMR ($CDCl_3$) indicated a mixture of compounds and TLC (mixture A) indicated three components with R_Fs 0.8 (UV active), 0.7, and 0.4 (Boc-Glyψ(NHCO)Gly-OEt, **99**)};

and Boc-Glyψ(NHCO)Gly-OEt, **99**, as a colourless solid (66 mg, 62%), identified by ^{1}H NMR and TLC (mixture A).

(c) N-*Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, with ethanolic hydrogen chloride*

Boc-Glyψ(NHCO)Gly-OEt, **99** (100 mg, 0.38 mmol) was dissolved in 5 M HCl in ethanol (2.0 mL) and stirred at r.t., in a dry atmosphere for 2 h. The solution was diluted with ethanol and evaporated to dryness to provide a yellow solid. Water was added to the solid and the resultant cloudy solution freeze dried to yield crude HCl.Glyψ(NHCO)Gly-OEt, **101**, as a hygroscopic yellow solid (91 mg, quantitative), m.p. 105-110°C; ν_{max} /cm^{-1} 3500-2500s (NH_3^+ and H_2O), 3255s (NH), 2000w (NH_3^+), 1735vs (ester carbonyl), 1680vs (amide carbonyl), 1545m (NH_3^+), 1535s (amide II), 1505m (NH_3^+), 1400m, 1345m, 1260m, 1195s, 1030m (C-O) and 950w; δ_H(270 MHz; $DMSO_{d6}$)‡ 1.19 (3 H, t, *J* 7.2, OCH_2CH_3), 3.36 (2 H, s, $COCH_2CO$), 4.08 (2 H, q, *J* 7.1, OCH_2CH_3), 4.22 (2 H, br d, $^+H_3NCH_2NH$), 8.35 (3 H, br s, ^+H_3N) and 9.24 (1 H, t, *J* 6.4, NH); δ_C(67.8 MHz; $DMSO_{d6}$) 14.3 (OCH_2CH_3), 42.3 ($COCH_2CO$), 44.7 ($^+H_3NCH_2NH$), 61.2 (OCH_2CH_3), 167.4 (CO) and 167.6 (CO); m/z 321 [$(2M-H)^+$, 21%], 161 (100, M^+, 161.0927, $C_6H_{13}N_2O_3$ requires 161.0926) and 132 (90, M - $HNCH_2$); m/z (-ve) 343 [$(^{37}Cl+2NBA)^-$, 11%], 341 [34, $(^{35}Cl+2NBA)^-$], 190 [34, $(^{37}Cl+NBA)^-$] and 188 [100, $(^{35}Cl+NBA)^-$]. Crystallisation of crude HCl.Glyψ(NHCO)Gly-OEt, **101**, from ethanol / ether, provided the pure compound as a colourless solid (28 mg, 0.14 mmol, 37%); δ_H(270 MHz; $DMSO_{d6}$) 1.19 (3 H, t, *J* 7.0, OCH_2CH_3), 3.33 (2 H, s, $COCH_2CO$), 4.08 (2 H, q, *J* 7.2, OCH_2CH_3), 4.22 (2 H, d, *J* 6.6, $^+H_3NCH_2NH$), 8.33 (3 H, br s, ^+H_3N) and 9.22 (1 H, t, *J* 6.4, NH).

(d) N-*Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, with TFA (in the presence of triethylsilane)*[243]

TFA (0.38 mL, 0.57 g, 5.0 mmol) and triethylsilane (0.15 mL, 112 mg, 0.96 mmol) were added to a solution of Boc-Glyψ(NHCO)Gly-OEt, **99** (100 mg, 0.38 mmol)

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‡ The crude material's ^{1}H NMR spectrum also contained a signal due to NH_4^+: 7.37 (0.5 H, t, $J_{H,N}$ 50.8).

in dichloromethane (0.8 mL). The mixture was stirred at r.t. in a dry atmosphere for 90 m, after which time TLC (mixture A) indicated all the starting material, **99**, was consumed. The mixture was evaporated to dryness and the residue triturated with ether. The resultant suspension was filtered, the precipitate washed with ether, dissolved in ethanol, evaporated and dried under high vacuum. The filtrate was evaporated, dried under high vacuum and further product obtained from the resultant residue by retrituration with ether, as before. The combined product, a colourless oil, was dissolved in water and freeze dried to yield TFA.Glyψ(NHCO)Gly-OEt, **102**, as a colourless oil which solidified on standing in a refrigerator to produce a hygroscopic colourless glassy solid (89 mg, 86%), m.p. 64-67°C (Found: C, 34.3; H, 4.75; N, 9.9. $C_8H_{13}N_2O_5F_3.{}^1/_3H_2O$ requires C: 34.3, H: 4.9, N: 10.0%); ν_{max} /cm^{-1} 3310s (NH), 3060br,s (NH_3^+), 2130w (NH_3^+), 1735vs (ester carbonyl), 1695vs (carboxylate), 1680vs (amide carbonyl), 1625s (NH_3^+), 1570s (NH_3^+), 1525s (amide II), 1430m, 1410m, 1340vs, 1310m, 1195br,vs (C-F and C-O), 1125vs (C-F or C-O), 1080w, 1025m, 965w, 950w, 875w, 840m, 800m, 725m (C-F), 685w (C-F) and 593m; δ_H(400 MHz; $DMSO_{d6}$) 1.19 (3 H, t, *J* 7.2, OCH_2CH_3), 3.35 (2 H, s, $COCH_2CO$), 4.09 (2 H, q, *J* 7.1, OCH_2CH_3), 4.25 (2 H, d, *J* 6.4, $^+H_3NCH_2NH$), 8.19 (3 H, br s, ^+H_3N) and 9.16 (1 H, br s, NH); δ_C(67.8 MHz; $DMSO_{d6}$) 14.2 (OCH_2CH_3), 42.3 ($COCH_2CO$), 44.7 ($^+H_3NCH_2NH$), 61.1 (OCH_2CH_3), 167.3 (CO) and 167.5 (CO); m/z 321 [$(2M-H)^+$, 44%], 183 [15, $(M-H+Na)^+$], 161 (56, M^+), 144 (20, M - NH_3) and 132 (100, M - $HNCH_2$); m/z (-ve) 341 [$(3CF_3CO_2+2H)^-$, 15%], 266 [11, $(CF_3CO_2+NBA)^-$], 249 [17, $(2CF_3CO_2+Na)^-$], 227 [69, $(2CF_3CO_2+H)^-$] and 113 (100, $CF_3CO_2^-$).

*(e) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-OEt, **99**, by saponification*

2 M Sodium hydroxide (15 mL) was added to a solution of Boc-Glyψ(NHCO)Gly-OEt, **99** (4.5 g, 17 mmol) in ethanol (90 mL). The mixture was stirred at r.t. overnight, then diluted with water and acidified to pH 2/3 by careful addition of 1 M potassium hydrogen sulfate. Brine (300 mL) was added and the solution extracted with ethyl acetate (5 x 200 mL). The organic extract was dried over sodium sulfate, evaporated and dried under high vacuum to yield Boc-Glyψ(NHCO)Gly, **103**, as a colourless solid (4.0 g, 99%), m.p. 121-126°C (Found: C, 46.4; H, 6.95; N, 11.9. $C_9H_{16}N_2O_5$ requires C, 46.55;

H, 6.95; N, 12.05%); ν_{max} /cm^{-1}§ 3355m (NH), 3320m (NH), 3100-2500br,w (CO_2H), 1720s (acid carbonyl), 1685m (Boc carbonyl), 1650vs (amide carbonyl), 1555w (amide II), 1535s (amide II), 1370w, 1275m, 1235m, 1160m, 1120m and 1050w; δ_H(270 MHz, $DMSO_{d6}$) 1.38 (9 H, s, Boc), 3.13 (2 H, s, $COCH_2CO$), 4.29 (2 H, t, *J* 6.1, $HNCH_2NH$), 7.45 (1 H, t, *J* 6.1, BocNH), 8.52 (1 H, br s, NH) and 12.49 (1 H, br s, CO_2H); δ_C(67.8 MHz, $DMSO_{d6}$) 28.3 [$(CH_3)_3C$], 42.4 ($COCH_2CO$), 45.3 ($HNCH_2NH$), 78.3 [$(CH_3)_3C$], 155.7 (Boc CO), 166.0 (amide CO) and 169.5 (acid CO); m/z 233 [$(M+H)^+$, 233.1143, $C_9H_{17}N_2O_5$ requires 233.1137, 37%], 177 [100, (M+H) - CH_2CMe_2], 116 (34, 177 - H_2O, CO, NH) and 104 [19, (M+H) - $BocNCH_2$].

*(f) Preparation of Boc-Glyψ(NHCO)Gly-OSu, **106***[242]

A solution of Boc-Glyψ(NHCO)Gly, **103** (84 mg, 0.36 mmol) and *N*-hydroxysuccinimide (42 mg, 0.36 mmol) in THF (8 mL) was cooled to 0°C. DCC (75 mg, 0.36 mmol) was added and the mixture stirred in a dry atmosphere at 0°C for 4 h, and then at r.t. overnight. The precipitated DCU was filtered off and washed with THF. The filtrate was evaporated and dried under high vacuum to yield crude Boc-Glyψ(NHCO)Gly-OSu, **106**, as a colourless solid (129 mg, quantitative). TLC (mixture F) of the crude material revealed the presence of starting materials and DCU. Recrystallisation of crude Boc-Glyψ(NHCO)Gly-OSu, **106**, from THF / petrol provided Boc-Glyψ(NHCO)Gly-OSu, **106**, as colourless crystals (29 mg, 24%); δ_H(270 MHz, $CDCl_3$, TMS)¶ 1.44 (9 H, s, Boc), 2.87 (4 H, s, succinimide H), 3.63 (2 H, s, $COCH_2CO$), 4.57 (2 H, t, *J* 6.2, $HNCH_2NH$), 5.59 (1 H, br s, BocNH) and 7.62 (1 H, br s, NH); m/z 330 [$(M+H)^+$, 49%], 274 [100, (M+H) - CH_2CMe_2], 225 [32, $(DCU+H)^+$], 213 (49, 274 -

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§ Nujol mull IR spectra of Boc-Glyψ(NHCO)Gly, **103**, showed great variation, particularly in the NH and CO absorptions, pressumably indicating the presence of more than one conformer and / or different hydrogen bonded species in varying ratios. Boc-Glyψ(NHCO)Gly, **103**, was insufficiently soluble in appropriate solvents (chloroform, dichloromethane and carbon disulfide) for the measurement of its solution phase IR spectra. Thus the ν_{max} values quoted are merely representative.

¶ The recrystallised material's ^{1}H NMR spectrum also contained the following signals, due to impurities: 1.00-1.40 (3 H, m, DCU H_{ax}), 1.50-2.00 (3 H, m, DCU H_{eq}) and 2.73 (0.5 H, s, HOSu CH_2).

H_2O, CO, NH), 201 [64, (M+H) - $BocNCH_2$], 177 (19), 138 (19), 116 [15, $(HOSu+H)^+$] and 83 (30, 201 - HOSu).

*(g) Coupling of Boc-Glyψ(NHCO)Gly-OSu, **106**, and HCl.Glyψ(NHCO)Gly-OEt, **101***

A solution of crude Boc-Glyψ(NHCO)Gly-OSu, **106** (290 mg, *ca.* 0.65 mmol*) in THF (20 mL) was added by decantation (leaving behind residual DCU) to crude HCl.Glyψ(NHCO)Gly-OEt, **101** (128 mg, *ca.* 0.65 mmol). Triethylamine (91 μL, 66 mg, 0.65 mmol) was added and the mixture stirred at r.t., in an inert atmosphere, for 5 days, after which time TLC (mixture E) indicated consumption of the Boc-Glyψ(NHCO)Gly-OSu, **106**, was complete. The cloudy solution was filtered, the colourless precipitate washed with water and collected. The filtrate was evaporated to dryness and the resultant yellow residue washed with water and the colourless precipitate collected. The combined precipitates were dried under high vacuum over phosphorus pentoxide. Column chromatography of the combined precipitates (preabsorbed onto silica from a suspension in ethyl acetate), eluting with chloroform / methanol / acetic acid (90:8:2) yielded Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, as a colourless solid [181 mg, 53% over the two steps from Boc-Glyψ(NHCO)Gly, **103**], m.p. 178-181°C (Found: C, 48.4; H, 7.1; N, 14.8. $C_{15}H_{26}N_4O_7$ requires C, 48.1; H, 7.0; N, 14.95%); ν_{max} / cm^{-1} 3320vs (NH), 1740m (ester carbonyl), 1690m (Boc carbonyl), 1675vs (amide carbonyl), 1650s (amide carbonyl), 1535s (amide II), 1530s (amide II), 1350w, 1280m, 1250w, 1230w, 1165w, 1150m, 1115m and 1035w; δ_H(400 MHz, $DMSO_{d6}$) 1.17 (3 H, t, *J* 7.2, OCH_2CH_3), 1.38 (9 H, s, Boc), 3.05 (2 H, s, $Gly^2C^{\alpha}H_2$), 3.22 (2 H, s, $Gly^4C^{\alpha}H_2$), 4.06 (2 H, q, *J* 7.1, OCH_2CH_3), 4.29 (2 H, t, *J* 6.0, $Gly^1C^{\alpha}H_2$), 4.38 (2 H, t, *J* 6.1, $Gly^3C^{\alpha}H_2$), 7.43 (1 H, br t, *J* 5.8, BocNH), 8.44 (1 H, br t, *J* 6.0, $Gly^1\psi NH$), 8.63 (1 H, br t, *J* 5.5, Gly^3NH) and 8.73 (1 H, br t, *J* 6.0, $Gly^3\psi NH$); δ_C(100.4 MHz, $DMSO_{d6}$) 14.1 (OCH_2CH_3), 28.3 [$(CH_3)_3C$], 42.2 ($COCH_2CO$), 42.9 ($COCH_2CO$), 43.5 ($HNCH_2NH$), 45.2 ($HNCH_2NH$), 60.5 (OCH_2CH_3), 78.2 [$(CH_3)_3C$], 155.7 (Boc CO), 165.8 (CO), 166.7 (CO), 167.3 (CO) and 167.7 (CO); m/z 375 $[(M+H)^+$, 23%], 319 [34, (M+H) - CH_2CMe_2], 258 (39, 319 -

* Estimated from ^{1}H NMR.

H_2O, CO, NH), 246 [31, (M+H) - $BocNCH_2$], 188 (25), 176 (28), 153 (25), 144 (20, $CH_2NHCOCH_2CO_2Et^+$), 132 [27, $H_2NC(OH)CH_2CO_2Et^+$], 127 (38), 115 (100, $COCH_2CO_2Et^+$), 107 (75), 103 (49) and 91 (51).

*(h) Coupling of Boc-Glyψ(NHCO)Gly, **103**, and HCl.Pro-Gly-OEt, **129***

A solution of Boc-Glyψ(NHCO)Gly, **103** (0.40 g, 1.7 mmol), HCl.Pro-Gly-OEt, **129** (0.45 g, 1.9 mmol) and *N*-hydroxysuccinimide (0.20 g, 1.7 mmol) in DMF (5 mL) was cooled to -10°C in a dry atmosphere. DCC (0.36 g, 1.7 mmol) and triethylamine (0.26 mL, 0.19 g, 1.9 mmol) were successively added at -10°C, with stirring. The mixture was stirred at -10°C for 4 h, and then at r.t. for a further 5 days. The precipitated DCU was filtered off and washed with dichloromethane (30 mL). The filtrate was evaporated to produce a brown residue which was dissolved in dichloromethane (100 mL) and the solution washed with 5% citric acid (50 mL) and water (50 mL plus a few drops of brine). The aq. layers were back-extracted with dichloromethane (50 mL), the combined organic extracts dried over sodium sulfate and evaporated. Column chromatography of the resultant solid (preabsorbed from dichloromethane solution), with chloroform / methanol (95:5) as the eluent, yielded Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130**, as a colourless solid (0.41 g, 58%); m.p. 122-124°C (Found: C, 52.1; H, 7.25; N, 13.4. $C_{18}H_{30}N_4O_7$ requires C, 52.15; H, 7.3; N, 13.5%); $[\alpha]_D^{16}$ - 62.5 (*c* 1.95 in dichloromethane); ν_{max} /cm^{-1} 3325s (NH), 1755w (ester carbonyl), 1730m (ester carbonyl), 1690s (Boc carbonyl), 1655vs (amide carbonyl), 1650vs (amide carbonyl), 1545s (amide II), 1520s (amide II), 1340w, 1305w, 1275w, 1255m, 1230w, 1195m, 1165m, 1115w, 1035w, 965w, 900w, 865w and 730w; δ_H(270 MHz, $CDCl_3$) 1.25 (3 H, t, *J* 7.1, OCH_2CH_3), 1.41 (9 H, s, Boc), 1.87-2.36 (4 H, m, $Pro^3C^\beta H_2$ and $C^\gamma H_2$), 3.32 (1 H, d, *J* 16.3, $Gly^2C^\alpha H_2$), 3.39 (1 H, d, *J* 16.3, $Gly^2C^\alpha H_2$), 3.47-3.76 (2 H, m, $Pro^3C^\delta H_2$), 3.92 (1 H, dd, *J* 5.4 and 18.0, $Gly^4C^\alpha H_2$), 4.05 (1 H, dd, *J* 5.8 and 18.0, $Gly^4C^\alpha H_2$), 4.17 (2 H, q, *J* 7.1, OCH_2CH_3), 4.51 (2 H, br t, *J* 6.4, $Gly^1C^\alpha H_2$), 4.61 (1 H, br dd, *J* 2.2 and 7.7, $Pro^3C^\alpha H$), 5.67 (0.8 H, br t, *J* 6.0, BocNH major conformer), 5.78 (0.2 H, br t, *J* 6.0, BocNH minor conformer), 7.32 (0.2 H, br s, NH minor conformer), 7.48 (0.8 H, br s, NH major conformer), 8.12 (0.7 H, br s, NH major conformer) and 8.24 (0.3 H, br s, NH minor conformer); δ_C(100.4

MHz; $CDCl_3$) 14.1 (OCH_2CH_3), 22.6 ($Pro^3C^{\gamma}H_2$ minor conformer), 24.6 ($Pro^3C^{\gamma}H_2$ major conformer), 28.2 [$(CH_3)_3C$], 28.5 ($Pro^3C^{\beta}H_2$ major conformer), 32.0 ($Pro^3C^{\beta}H_2$ minor conformer), 41.2 ($Gly^4C^{\alpha}H_2$), 42.1 ($Gly^2C^{\alpha}H_2$), 46.1 ($Gly^1C^{\alpha}H_2$), 47.2 ($Pro^3C^{\delta}H_2$ minor conformer), 47.9 ($Pro^3C^{\delta}H_2$ major conformer), 59.9 ($Pro^3C^{\alpha}H$ major conformer), 61.1 ($Pro^3C^{\alpha}H$ minor conformer), 61.3 (OCH_2CH_3 major conformer), 61.7 (OCH_2CH_3 minor conformer), 79.9 [$(CH_3)_3C$], 155.7 (Boc CO), 167.3 (amide CO), 167.4 (amide CO), 169.9 (Gly^4CO_2Et) and 171.3 (Pro^3CO); m/z 415 [$(M+H)^+$, 40%], 359 [24, (M+H) - CH_2CMe_2], 298 (46, 359 - H_2O, CO, NH), 286 [100, (M+H) - $BocNCH_2$], 269 (22, 298 - H_2CNH), 228 (13), 201 [16, (Pro-Gly-OEt+H)$^+$], 199 (23), 183 (17), 112 (10) and 73 (13).

(i) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, ***130****, by saponification*

2 M Sodium hydroxide (0.9 mL) was added to a solution of Boc-Glyψ(NHCO)Gly-Pro-Gly-OEt, **130** (373 mg, 0.90 mmol) in ethanol (5 mL). The mixture was stirred at r.t. overnight, then diluted with water and acidified to pH 2/3 by careful addition of 1 M potassium hydrogen sulfate. Brine (20 mL) was added and the solution extracted with dichloromethane (10 x 20 mL). The combined organic extracts were dried over sodium sulfate, evaporated and dried under high vacuum. The resultant colourless solid was dissolved in water and freeze-dried to yield Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**, as a colourless solid (304 mg, 88%), m.p. 142-148°C (change in appearance noted at 45-65°C); $[\alpha]_D^{20}$ - 78.5 (*c* 0.11 in water); ν_{max} /cm^{-1} 3330vs (NH), 3200-2500br,w (CO_2H), 1725m (acid carbonyl), 1680m (Boc carbonyl), 1660s (amide carbonyl), 1650vs (amide carbonyl), 1620vs (amide carbonyl), 1550m (amide II), 1535m (amide II), 1525m (amide II), 1340m, 1325m, 1285m, 1255m, 1220m, 1150s, 1115m, 1030w, 970w, 900w, 865w, 800w, 775w, 725w, 680w and 650w; δ_H(270 MHz, $DMSO_{d6}$) 1.38 (9 H, s, Boc), 1.70-2.23 (4 H, m, $Pro^3C^{\beta}H_2$ and $C^{\gamma}H_2$), 3.00 (0.3 H, d, *J* 15.4, $Gly^2C^{\alpha}H_2$ minor conformer), 3.17 (0.5 H, d, *J* 15.4, $Gly^2C^{\alpha}H_2$ minor conformer), 3.26 (1.2 H, s,

$Gly^2C^{\alpha}H_2$ major conformer), 3.29-3.60 (3 H‡, m, $Pro^3C^{\delta}H_2$), 3.66 (1 H, dd, *J* 5.9 and 17.6, $Gly^4C^{\alpha}H_2$), 3.75 (1 H, dd, *J* 5.7 and 17.4, $Gly^4C^{\alpha}H_2$), 4.30 (2.7 H, m, $Gly^1C^{\alpha}H_2$ and $Pro^3C^{\alpha}H$ major conformer), 4.53 (0.3 H, dd, *J* 3.0 and 6.2, $Pro^3C^{\alpha}H$ minor conformer), 7.45 (1 H, m, BocNH)§, 8.16 (0.6 H, t, *J* 5.9, NH major conformer), 8.44 (0.3 H, t, *J* 6.0, NH minor conformer), 8.51 (0.4 H, t, *J* 6.0, NH minor conformer)¶, 8.59 (0.7 H, t, *J* 5.3, NH major conformer)¶ and 12.55 (0.4 H, br s, CO_2H); δ_C(100.4 MHz; $DMSO_{d6}$) 22.4 ($Pro^3C^{\gamma}H_2$ minor conformer), 23.9 ($Pro^3C^{\gamma}H_2$ major conformer), 28.3 [$(CH_3)_3C$], 29.7 ($Pro^3C^{\beta}H_2$ major conformer), 31.6 ($Pro^3C^{\beta}H_2$ minor conformer), 40.6 ($Gly^4C^{\alpha}H_2$ major conformer), 40.9 ($Gly^4C^{\alpha}H_2$ minor conformer), 42.0 ($Gly^2C^{\alpha}H_2$ minor conformer), 42.5 ($Gly^2C^{\alpha}H_2$ major conformer), 45.3 ($Gly^1C^{\alpha}H_2$), 46.5 ($Pro^3C^{\delta}H_2$ minor conformer), 47.3 ($Pro^3C^{\delta}H_2$ major conformer), 59.5 ($Pro^3C^{\alpha}H$ major conformer), 60.2 ($Pro^3C^{\alpha}H$ minor conformer), 78.3 [$(CH_3)_3C$], 155.7 (Boc CO), 166.4 (amide CO major conformer), 166.5 (amide CO minor conformer), 166.5 (amide CO minor conformer), 166.8 (amide CO major conformer), 171.2 (Gly^4CO_2H minor conformer), 171.2 (Gly^4CO_2H major conformer), 171.9 (Pro^3CO major conformer) and 172.2 (Pro^3CO minor conformer); m/z (FAB, glycerol) 387 [$(M+H)^+$, 387.1860, $C_{16}H_{27}N_4O_7$ requires 387.1880, 32%], 331 [11, (M+H) - CH_2CMe_2], 270 (18, 331 - H_2O, CO, NH), 258 [34, (M+H) - $BocNCH_2$], 239 (12), 155 {15, $H_2NCOCH_2CO\overline{N[CH_2]_3}\overline{CH}^+$}, 147 (17), 91 (19), 70 (42), 61 (14), 57 (36, Bu^{t+}), 45 (63), 43 (29), 31 (58, CH_2OH^+) and 29 (100, CHO^+).

*(j) Coupling of Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**, and TFA.Glyψ(NHCO)Gly-OEt, **102***

A solution of TFA.Glyψ(NHCO)Gly-OEt, **102** (92 mg, 0.34 mmol) in DMF (2.0 mL) was prepared. A solution of Boc-Glyψ(NHCO)Gly-Pro-Gly, **131** (115 mg, 0.30 mmol) in DMF (1.0 mL) was cooled to -15°C in an inert atmosphere. *N*-Methylmorpholine (33 μL, 30 mg, 0.30 mmol) and *iso*-butylchloroformate (39 μL, 41 mg,

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‡ Due to presence of water signal.

§ At 400 MHz this signal appeared to be two overlapping triplets: δ_H 7.40 (*J* 5.9) and 7.44 (*J* 5.9).

¶ At 100°C these two signals virtually coalesced.

0.30 mmol) were successively added and stirring continued at -15°C, in an inert atmosphere, for 30 m.

Triethylamine (47 μL, 34 mg, 0.34 mmol) was added to the solution of TFA.Glyψ(NHCO)Gly-OEt, **102**, and the resultant mixture immediately added to the solution of Boc-Glyψ(NHCO)Gly-Pro-Gly, **131**, *N*-methylmorpholine and *iso*-butylchloroformate. Stirring was continued at -15°C for 30 m, and then at r.t. overnight, in an inert atmosphere. The solution was evaporated to produce an off-white residue, to which water (10 mL) was added. The resultant colourless precipitate was collected, washed with water (10 mL) and dried under high vacuum over phosphorus pentoxide. The filtrate was freeze-dried and further crude product recovered from the resultant colourless solid by the addition of dichloromethane (20 mL) and water (30 mL); the layers were separated and the aq. phase back-extracted with dichloromethane (4 x 20 mL). The combined organic extracts were dried over sodium sulfate and evaporated. The two portions of crude product were combined and column chromatography (sample applied as a slurry in the initial eluent), with chloroform / methanol / acetic acid (95:3:2 then 90:8:2) as the eluent, yielded Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132**, as a colourless solid (55 mg, 35%); m.p. 172-177°C; $[\alpha]_D^{18}$ - 20.6 (*c* 0.76 in TFE); ν_{max} /cm^{-1} 3315s (NH), 3075w (NH), 1750m (ester carbonyl), 1690s (Boc carbonyl), 1655vs (amide carbonyl), 1650vs (amide carbonyl), 1635vs (amide carbonyl), 1545s (amide II), 1525s (amide II), 1345w, 1280m, 1230m, 1165m, 1120m, 1040w, 970w, 900w and 720w; δ_H(270 MHz, $DMSO_{d6}$; TMS) 1.17 (3 H, t, *J* 7.1, OCH_2CH_3), 1.38 (9 H, s, Boc), 1.70-2.20 (4 H, m, Pro^{3}C$^\beta$H$_2$ and C$^\gamma$H$_2$), 3.00-3.31 (4 H, m, Gly^{2}C$^\alpha$H$_2$ and Gly^{6}C$^\alpha$H$_2$), 3.39-3.59 (2 H, m, Pro^{3}C$^\delta$H$_2$), 3.56-3.75 (2 H, m, Gly^{4}C$^\alpha$H$_2$)*, 4.06 (2 H, q, *J* 7.1, OCH_2CH_3), 4.23-4.36 (2.5 H, m, Gly^{1}C$^\alpha$H$_2$ and Pro^{3}C$^\alpha$H major conformer), 4.40 (2 H, t, *J* 6.0, Gly^{5}C$^\alpha$H$_2$), 4.52 (0.5 H, br d, *J* 8.4, Pro^{3}C$^\alpha$H minor conformer), 7.44 (1 H, m, BocNH), 8.16 (0.7 H, t, *J* 6.0, Gly^{4}NH major conformer), 8.29 (0.3 H, t, *J* 6.0, Gly^{4}NH minor conformer), 8.36 (0.7 H, t, *J* 6.0, Gly^{5}NH major conformer), 8.52 (0.3 H, t, *J* 6.4,

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* This signal appeared to be two doublets of doublets [δ_H 3.62 (dd, *J* 6.0 and 16.7) and 3.69 (dd, *J* 5.9 and 16.7)] due to the major conformer, superimposed on a multiplet due to the minor conformer.

Gly$^1\psi$NH minor conformer), 8.59 (0.9 H, m, Gly$^1\psi$NH major conformer and Gly^{5}NH minor conformer), 8.68 (0.7 H, t, *J* 6.0, Gly$^5\psi$NH major conformer) and 8.74 (0.3 H, t, *J* 5.8, Gly$^5\psi$NH minor conformer); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 ($OCH_2\mathit{C}H_3$), 22.4 (Pro$^3C^\gamma H_2$ minor conformer), 24.1 (Pro$^3C^\gamma H_2$ major conformer), 28.3 [$(\mathit{C}H_3)_3C$], 29.5 (Pro$^3C^\beta H_2$ major conformer), 31.6 (Pro$^3C^\beta H_2$ minor conformer), 41.7 (Gly$^4C^\alpha H_2$ minor conformer), 41.8 (Gly$^4C^\alpha H_2$ major conformer), 42.2 ($CO\mathit{C}H_2CO$), 42.5 ($CO\mathit{C}H_2CO$), 43.4 ($HNCH_2NH$ minor conformer), 43.5 ($HNCH_2NH$ major conformer), 45.3 ($HNCH_2NH$), 46.5 (Pro$^3C^\delta H_2$ minor conformer), 47.4 (Pro$^3C^\delta H_2$ major conformer), 59.8 (Pro$^3C^\alpha H$ major conformer), 60.3 (Pro$^3C^\alpha H$ minor conformer), 60.5 ($O\mathit{C}H_2CH_3$), 78.3 [$(CH_3)_3\mathit{C}$], 155.7 (Boc CO), 165.8 (CO), 165.8 (CO minor conformer), 166.6 (CO), 166.8 (CO), 167.8 (CO), 169.4 (Gly^{4}CO), 171.8 (Pro^{3}CO major conformer) and 172.1 (Pro^{3}CO minor conformer); m/z 551 [$(M+Na)^+$, 68%], 529 [100, $(M+H)^+$, 529.2632, $C_{22}H_{37}N_6O_9$ requires 529.2622], 473 [9, (M+H) - CH_2CMe_2], 412 (19, 473 - H_2O, CO, NH), 400 [46, (M+H) - $BocNCH_2$], 313 (19), 269 (17, H_2NCOCH_2CO-Pro-Gly-$NHCH_2^+$), 257 [36, H_2NCOCH_2CO-Pro-$NHCH_2C(OH)NH_2^+$], 240 (46, H_2NCOCH_2CO-Pro-$NHCH_2CO^+$) and 183 {23, $H_2NCOCH_2CO\overline{N[CH_2]_3}CHCO^+$}.

(k) N-*Terminal deprotection of Boc-Gly*ψ*(NHCO)Gly-Gly*ψ*(NHCO)Gly-OEt,* ***104****, with ethanolic hydrogen chloride*

Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** (61 mg, 0.16 mmol) was dissolved in 5 M HCl in ethanol (5.0 mL) and stirred at r.t., in a dry atmosphere for 4 h. The solution was diluted with ethanol and evaporated to dryness to provide an off-white residue. Water was added to the residue and the resultant cloudy solution freeze dried to yield crude HCl.Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **139**, as a hygroscopic off-white solid (56 mg, quantitative); δ_H(270 MHz; $DMSO_{d6}$)† 1.17 (3 H, t, *J* 7.0, $OCH_2C\mathit{H}_3$), 3.18 (2 H, s, $COCH_2CO$), 3.24 (2 H, s, $COCH_2CO$), 4.06 (2 H, q, *J* 7.1, $OC\mathit{H}_2CH_3$), 4.21 (2 H, br m, $^+H_3NC\mathit{H}_2NH$), 4.40 (2 H, t, $HNC\mathit{H}_2NH$), 8.26 (3 H, br s, ^+H_3N), 8.76 (2 H, m, 2xNH) and 9.04 (1 H, t, *J* 6.0, NH).

† The crude material's ^{1}H NMR spectrum also contained the following signals, due to impurities: 3.15 (1 H, s) and 7.32 (1 H, t, $J_{H,N}$ 50.7, NH_4^+).

(l) N-*Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt,* ***104****, with TFA*

TFA (0.13 mL, 192 mg, 1.7 mmol) was added to a suspension of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** (50 mg, 0.13 mmol) in dichloromethane (0.2 mL). The mixture was stirred at r.t. in a dry atmosphere for 4 h, after which time TLC (mixture D) indicated all the starting material, **104**, was consumed. The mixture was evaporated to dryness, petrol (2 mL) added and the mixture slowly stirred overnight. The petrol was decanted off and the process repeated with a further aliquot of petrol (2 mL). The resultant off-white residue was triturated with ether. The ether was decanted off and the process repeated with a further aliquot of ether. The resultant residue was dried under high vacuum to yield TFA.Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **137**, as a hygroscopic beige solid (40 mg, 77%), m.p. 121-127°C dec; ν_{max} /cm^{-1} 3600-2400br,m (NH_3^+ and H_2O), 3295vs (NH), 1965br,w (NH_3^+), 1740br,s (ester carbonyl), 1730s (ester carbonyl), 1715m, 1705w, 1700s, 1695s (carboxylate), 1680s (amide carbonyl), 1675w (amide carbonyl), 1665m (amide carbonyl), 1660m (amide carbonyl), 1650vs (amide carbonyl), 1645vs (amide carbonyl), 1635s (amide carbonyl), 1620w (NH_3^+), 1615w (NH_3^+), 1575w (amide II), 1565w (amide II), 1555s (amide II), 1540s (amide II), 1115m, 975br,m, 840br,m, 725vs (C-F) and 665vs (C-F); δ_H(400 MHz; $DMSO_{d6}$; TMS) 1.18 (3 H, t, *J* 7.0, OCH_2CH_3), 3.19 (2 H, s, $COCH_2CO$), 3.24 (2 H, s, $COCH_2CO$), 4.07 (2 H, q, *J* 7.0, OCH_2CH_3), 4.22 (2 H, d, *J* 6.1, $^+H_3NCH_2NH$), 4.41 (2 H, br t, *J* 5.5, $HNCH_2NH$), 8.23 (3 H, br s, ^+H_3N), 8.76 (2 H, m, 2xNH) and 9.02 (1 H, br t, *J* 6.0, NH); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 42.2 ($COCH_2CO$), 42.8 ($COCH_2CO$), 43.6 ($HNCH_2NH$), 44.6 ($HNCH_2NH$), 60.5 (OCH_2CH_3), 165.9 (CO), 166.8 (CO), 167.7 (CO) and 168.2 (CO); m/z 549 [$(2M-H)^+$, 12%], 393 (17), 322 (15), 297 [10, $(M-H+Na)^+$], 275 (100, 275.1340, $C_{10}H_{19}N_4O_5$ requires 275.1355, M^+), 258 (23, M - NH_3), 246 (47, M - $HNCH_2$), 176 (12), 144 (12, $H_2CNHCOCH_2CO_2Et^+$), 132 (15), 127 (13) and 103 [17, $H_2NCOCH_2C(OH)NH_2^+$].

*(m) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104**, by saponification*

2 M Sodium hydroxide (0.6 mL) was added to a suspension of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** (232 mg, 0.62 mmol) in ethanol (8 mL). The mixture was stirred at r.t. overnight, then diluted with water and the cloudy solution acidified to pH 2/3 by careful addition of Amberlite IR 120(+). The supernatant liquid (and the suspended colourless solid) was decanted off (leaving behind the ion-exchange resin) and freeze-dried to yield Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly, **135**, as a colourless solid (191 mg, 89%), m.p. 128-140°C dec; ν_{max} /cm^{-1} 3355m (NH), 3300s (NH), 3150-2500br,w (CO_2H), 1720m (acid carbonyl), 1695s (Boc carbonyl), 1655vs (amide carbonyl), 1650vs (amide carbonyl), 1640vs (amide carbonyl), 1545s (amide II), 1530s (amide II), 1345w, 1320w, 1280m, 1255w, 1235m, 1170m, 1120m and 740w; δ_H(270 MHz, $DMSO_{d6}$) 1.38 (9 H, s, Boc), 3.05 (2 H, s, $COCH_2CO$), 3.13 (2 H, s, $COCH_2CO$), 4.29 (2 H, t, *J* 5.7, $HNCH_2NH$), 4.38 (2 H, t, *J* 5.9, $HNCH_2NH$), 7.44 (1 H, br t, *J* 6.0, BocNH), 8.45 (1 H, br t, *J* 6.0, NH), 8.63 (1 H, br t, *J* 6.0, NH), 8.70 (1 H, br t, *J* 5.5, NH) and 12.45 (1 H, br s, CO_2H); δ_C(100.4 MHz, $DMSO_{d6}$) 28.3 [$(CH_3)_3C$], 42.4 ($COCH_2CO$), 42.9 ($COCH_2CO$), 43.5 ($HNCH_2NH$), 45.2 ($HNCH_2NH$), 78.3 [$(CH_3)_3C$], 155.7 (Boc CO), 166.4 (amide CO), 166.7 (amide CO), 167.3 (amide CO) and 169.3 (acid CO); m/z (FAB, glycerol) 439 [(M+H+glycerol)$^+$, 11%], 347 [100, 347.1594, $C_{13}H_{23}N_4O_7$ requires 347.1567, (M+H)$^+$], 291 [60, (M+H) - CH_2CMe_2], 230 (31, 291 - H_2O, CO, NH), 218 [57, (M+H) - $BocNCH_2$], 188 (31), 176 (49), 147 (12), 127 (17), 115 (29, $H_2NCOCH_2CONHCH_2^+$), 103 [23, $H_2NCOCH_2C(OH)NH_2^+$], 91 (15), 86 (12), 57 (35, Bu^{t+}) and 30 (19, $H_2NCH_2^+$).

*(n) Coupling of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly, **135**, and HCl.Pro-Gly-OEt, **129***

A solution of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly, **135** (178 mg, 0.52 mmol) and HCl.Pro-Gly-OEt, **129** (246 mg, 1.0 mmol) in DMF (4 mL) was cooled to -10°C in a dry atmosphere. *N*-Hydroxysuccinimide (60 mg, 0.5 mmol), EDC.HCl (199 mg, 1.0 mmol) and triethylamine (290 μL, 210 mg, 2.1 mmol) were successively added at -10°C, with stirring. The mixture was stirred at -10°C for 4 h, and then at r.t. for a further 4 days.

The solution was evaporated to dryness and water (10 mL) added to the resultant yellow residue: very little product precipitated, so the mixture was freeze-dried. Column chromatography [sample applied as a slurry in chloroform / methanol / acetic acid (95:3:2)], with chloroform / methanol / acetic acid (90:8:2) as the eluent, followed by washing of the collected product with petrol and drying under high vacuum, yielded Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, as a colourless solid (55 mg, 20%); m.p. 176-179°C dec; $[\alpha]_D^{20}$ - 58.5 (*c* 0.96 in TFE); ν_{max} /cm^{-1} 3340br,s (NH), 3310br,s (NH), 1730m (ester carbonyl), 1695s (Boc carbonyl), 1650vs (amide carbonyl), 1635vs (amide carbonyl), 1555m (amide II), 1540s (amide II), 1520s (amide II), 1505s (amide II), 1340m, 1295br,m, 1230m, 1165m, 1115m, 1030m, 1005m, 960w, 940w and 720w; δ_H(400 MHz, $DMSO_{d6}$)‡ 1.18 (3 H, br t, *J* 7.1, OCH_2CH_3), 1.38 (9 H, s, Boc), 1.74-2.26 (5 H§, br m, Pro$^5C^\beta H_2$ and $C^\gamma H_2$), 2.97-3.29 (5 H¶, m, Gly$^2C^\alpha H_2$ and Gly$^4C^\alpha H_2$), 3.40-3.60 (2 H, m, Pro$^5C^\delta H_2$), 3.71-3.87 (2 H, m, Gly$^6C^\alpha H_2$)*, 4.08 (2 H, m, OCH_2CH_3)†, 4.29 (2.7 H, m, Gly$^1C^\alpha H_2$ and Pro$^5C^\alpha H$ major conformer), 4.40 (2 H, m, Gly$^3C^\alpha H_2$), 4.52 (0.3 H, dd, *J* 3.0 and 8.0, Pro$^5C^\alpha H$ minor conformer), 7.42 (1 H, t, *J* 6.0, BocNH), 8.25 (0.7 H, t, *J* 5.7, Gly^{6}NH major conformer), 8.44 (1 H, br s, Gly^{1}ψNH), 8.54 (0.3 H, t, *J* 5.5, Gly^{6}NH minor conformer), 8.61 (1 H, t, *J* 6.1, Gly^{3}NH), 8.68 (0.2 H, t, *J* 6.0, Gly^{3}ψNH minor conformer) and 8.74 (0.5 H, t, *J* 6.0, Gly^{3}ψNH major conformer); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 22.2 (Pro$^5C^\gamma H_2$ minor conformer), 23.9 (Pro$^5C^\gamma H_2$ major conformer), 28.3 [$(CH_3)_3C$], 29.7 (Pro$^5C^\beta H_2$ major conformer), 31.6 (Pro$^5C^\beta H_2$ minor conformer), 40.7 (Gly$^6C^\alpha H_2$ major conformer), 40.9 (Gly$^6C^\alpha H_2$ minor

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‡ Accumulation at 50°C resulted in little change in the spectrum.

§ There is no obvious source of the extra proton.

¶ Extra proton due to overlap with the water signal.

* This signal appeared to be two doublets of doublets: δ_H 3.75 (dd *J* 6.0 and 17.5) and 3.83 (dd, *J* 6.0 and 17.2); with an overlapping multiplet, presumably due to the minor conformer.

† This signal consisted of two overlapping quartets: δ_H 4.07 (q, *J* 7.1, major conformer) and 4.08 (q, *J* 7.0, minor conformer).

conformer), 41.9 ($COCH_2CO$ minor conformer), 42.4 ($COCH_2CO$ major conformer), 42.9 ($COCH_2CO$), 43.5 ($HNCH_2NH$), 45.2 ($HNCH_2NH$), 46.5 ($Pro^5C^{\delta}H_2$ minor conformer), 47.3 ($Pro^5C^{\delta}H_2$ major conformer), 59.4 ($Pro^5C^{\alpha}H$ major conformer), 60.4 ($Pro^5C^{\alpha}H$ minor conformer), 60.5 (OCH_2CH_3 major conformer), 60.6 (OCH_2CH_3 minor conformer), 78.3 [$(CH_3)_3C$], 155.7 (Boc CO), 166.2 (amide CO), 166.7 (amide CO), 167.3 (amide CO), 167.3 (amide CO), 169.8 (Gly^6CO_2Et), 172.1 (Pro^5CO major conformer) and 172.4 (Pro^5CO minor conformer); m/z 1057 [$(2M+H)^+$, 18%], 652 (16), 551 [54, $(M+Na)^+$], 529 [95, $(M+H)^+$, 529.2627, $C_{22}H_{37}N_6O_9$ requires 529.2622], 412 [35, (M+H) - CH_2CMe_2, H_2O, CO, NH], 400 [95, (M+H) - $BocNCH_2$], 298 (72, $H_2CNHCOCH_2CO$-Pro-Gly-OEt$^+$), 286 [100, $H_2NC(OH)CH_2CO$-Pro-Gly-OEt$^+$], 269 (42, $OCCH_2CO$-Pro-Gly-OEt$^+$), 201 [27, (Pro-Gly-OEt+H)$^+$], 199 (21) and 182 (23).

*(o) C-Terminal deprotection of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136**, by saponification*

2 M Sodium hydroxide (0.1 mL) was added to a suspension of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-OEt, **136** (18.6 mg, 0.035 mmol) in ethanol (2 mL). The mixture was stirred at r.t. overnight, then diluted with water and the cloudy solution acidified to pH 2/3 by careful addition of Amberlite IR 120(+). The supernatant liquid (and the suspended colourless solid) was decanted off (leaving behind the ion-exchange resin) and freeze-dried to yield Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly, **138**, as an off-white solid (14.7 mg, 84%), m.p. 133-182°C dec (change in appearance noted at 102-110°C); $[\alpha]_D^{21}$ - 53.0 (*c* 0.41 in TFE); ν_{max} /cm^{-1} 3305br,m (NH), 1730w (acid carbonyl), 1690m (Boc carbonyl), 1665s (amide carbonyl), 1655s (amide carbonyl), 1650vs (amide carbonyl), 1645vs (amide carbonyl), 1535m (amide II), 1525m (amide II), 1170m, 1120m and 720s; δ_H(270 MHz, $DMSO_{d6}$; TMS) 1.39 (9 H, s, Boc), 1.71-2.08 (4 H, m, $Pro^5C^{\beta}H_2$ and $C^{\gamma}H_2$), 2.92-3.29 (4 H, m, $Gly^2C^{\alpha}H_2$ and $Gly^4C^{\alpha}H_2$), 3.43-3.61 (2 H, m, $Pro^5C^{\delta}H_2$), 3.68 (1 H, dd, *J* 6.0 and 18.0, $Gly^6C^{\alpha}H_2$), 3.76 (1 H, dd, *J* 6.0 and 16.5, $Gly^6C^{\alpha}H_2$), 4.29 (2.7 H, m, $Gly^1C^{\alpha}H_2$ and $Pro^5C^{\alpha}H$ major conformer), 4.40 (2 H, t, *J* 5.3, $Gly^3C^{\alpha}H_2$), 4.51 (0.3 H, dd, *J* 3.0 and 9.0, $Pro^5C^{\alpha}H$ minor conformer), 7.44 (1 H, t, *J* 6.0, BocNH), 8.16 (1 H, t, *J* 6.0, NH), 8.46 (1 H, br m, NH), 8.63 (1 H, m,

NH), 8.75 (1 H, t, *J* 6.0, NH) and 12.40 (1 H, br s, CO_2H); δ_C(100.4 MHz; $DMSO_{d6}$) 22.7 ($Pro^5C^{\gamma}H_2$ minor conformer), 23.9 ($Pro^5C^{\gamma}H_2$ major conformer), 28.3 [$(CH_3)_3C$], 29.7 ($Pro^5C^{\beta}H_2$ major conformer), 31.9 ($Pro^5C^{\beta}H_2$ minor conformer), 40.7 ($Gly^6C^{\alpha}H_2$ major conformer), 40.9 ($Gly^6C^{\alpha}H_2$ minor conformer), 42.2 ($COCH_2CO$ minor conformer), 42.4 ($COCH_2CO$ major conformer), 42.9 ($COCH_2CO$), 43.5 ($HNCH_2NH$), 45.2 ($HNCH_2NH$), 46.5 ($Pro^5C^{\delta}H_2$ minor conformer), 47.3 ($Pro^5C^{\delta}H_2$ major conformer), 59.5 ($Pro^5C^{\alpha}H$ major conformer), 60.2 ($Pro^5C^{\alpha}H$ minor conformer), 78.3 [$(CH_3)_3C$], 156.0 (Boc CO), 166.2 (amide CO), 167.1 (br, amide CO), 167.3 (amide CO), 167.3 (amide CO), 171.2 (Gly^6CO_2H), 171.9 (Pro^5CO major conformer) and 172.1 (Pro^5CO minor conformer); m/z 523 [$(M+Na)^+$, 71%], 501 [56, 501.2291, $C_{20}H_{33}N_6O_9$ requires 501.2309, $(M+H)^+$], 392 (40), 384 [26, (M+H) - CH_2CMe_2, H_2O, CO, NH], 372 [69, (M+H) - $BocNCH_2$], 322 (24), 270 (81, $H_2CNHCOCH_2CO$-Pro-Gly^+), 258 [100, $H_2NC(OH)CH_2CO$-Pro-Gly^+], 241 (24, $OCCH_2CO$-Pro-Gly^+), 214 (13), 199 (16), 195 (13), 188 (18), 183 (11), 176 (35), 173 [26, $(Pro\text{-}Gly+H)^+$], 171 (21), 127 (34), 115 (80, $H_2NCOCH_2CONHCH_2^+$), 113 (46), 105 (33), 103 [23, $H_2NCOCH_2C(OH)NH_2^+$], 91 (49) and 86 (27).

(p) N-*Terminal deprotection of Boc-Gly*ψ*(NHCO)Gly,* ***103****, with TFA*

TFA (3.2 mL, 4.7 g, 42 mmol) was added to a solution of Boc-Glyψ(NHCO)Gly, **103** (734 mg, 3.2 mmol) in dichloromethane (5.3 mL). The mixture was stirred at r.t. in a dry atmosphere for 150 m, after which time TLC (mixture E) indicated all the starting material, **103**, was consumed. The solution was evaporated to dryness, petrol (30 mL) added to the residue and the resultant mixture slowly stirred overnight. The petrol was decanted off and the process repeated with a further aliquot of petrol (30 mL). The resultant colourless residue was dissolved in ethanol, evaporated and dried under high vacuum to yield TFA.Glyψ(NHCO)Gly, **141**, as a colourless solid (787 mg, quantitative), m.p. 120-125°C dec (Found: C, 29.2; H, 3.55; N, 11.1. $C_6H_9N_2O_5F_3$ requires C: 29.3, H: 3.7, N: 11.4%); ν_{max} /cm^{-1} 3300m (NH), 3060br,m (CO_2H and NH_3^+), 2690br,w (CO_2H and NH_3^+), 2570br,w (CO_2H and NH_3^+), 1720s (acid carbonyl), 1680vs (carboxylate), 1660vs (amide carbonyl), 1595m (NH_3^+), 1550s (amide II), 1500w (NH_3^+), 1430w, 1410w, 1330s, 1305w, 1220s (C-F and / or C-O), 1200vs (C-F and / or C-O), 1140s (C-F

and / or C-O), 1130m (C-F and / or C-O), 1085w, 1000w, 950w, 930w, 910w, 855w, 845m, 805w, 785w and 725m (C-F); δ_H(270 MHz; $DMSO_{d6}$) 3.25 (2 H, s, $COCH_2CO$), 4.25 (2 H, d, *J* 6.2, $^+H_3NCH_2NH$), 8.20 (3 H, br s, ^+H_3N), 9.11 (1 H, t, *J* 6.4, NH) and 12.83 (1 H, br s, CO_2H); δ_C(67.8 MHz; $DMSO_{d6}$) 42.4 ($COCH_2CO$), 44.8 ($^+H_3NCH_2NH$), 167.4 (amide CO) and 168.4 (acid CO); m/z 286 [$(M+NBA)^+$, 27%], 133 (100, M^+), 116 (14, M - NH_3) and 94 (12); m/z (-ve) 266 [$(CF_3CO_2+NBA)^-$, 72%] and 113 (100, $CF_3CO_2^-$).

(q) N-*Terminal protection of TFA.Glyψ(NHCO)Gly,* ***141****, with Fmoc*[333]

TFA.Glyψ(NHCO)Gly, **141** (703 mg, 2.9 mmol) and sodium hydrogen carbonate (480 mg, 5.7 mmol) were dissolved in a mixture of water (9 mL) and acetone (9 mL) with a concomitant evolution of gas. 9-Fluorenylmethyloxycarbonyl succinimide (963 mg, 2.9 mmol) was added and the cloudy mixture stirred overnight at r.t., after which time TLC (mixture E) indicated complete consumption of the 9-fluorenylmethyloxycarbonyl succinimide. The mixture was diluted with acetone and acidified to pH 2 with 2 M hydrochloric acid. The solution was concentrated *in vacuo*, the precipitate collected, washed with water (100 mL) and dried under high vacuum over phosphorus pentoxide. Column chromatography (sample applied as a slurry in the initial eluent) with chloroform / methanol / acetic acid (95:3:2 then 90:8:2) yielded crude Fmoc-Glyψ(NHCO)Gly, **143**, as a colourless solid (928 mg, 92%) [crystallisation from ethanol yielded Fmoc-Glyψ(NHCO)Gly, **143**, free from impurities, but with retained ethanol, which would cause problems during the SPS; therefore Fmoc-Glyψ(NHCO)Gly, **143**, was largely characterised without crystallisation], m.p. 182-185°C (from ethanol) (Found: C, 63.0; H, 5.6; N, 7.1. $C_{19}H_{18}N_2O_5.0.8EtOH$ requires C: 63.25, H: 5.85, N: 7.15%); ν_{max} /cm^{-1} 3315vs (NH), 3000-2500br,w (CO_2H), 1725s (acid carbonyl), 1700s (Fmoc CO), 1660vs (amide carbonyl), 1545m (amide II), 1530s (amide II), 1340w, 1315m, 1265s, 1230m, 1170w, 1120m, 1050w, 985m, 900w, 785w, 760m and 740m; δ_H(400 MHz; $DMSO_{d6}$;

TMS)† 3.16 (2 H, s, $COCH_2CO$), 4.19-4.29 (3 H, m, Fmoc CH and CH_2), 4.38 (2 H, t, *J* 5.9, $HNCH_2NH$), 7.33 (2 H, t, *J* 7.3, Fmoc Ar CH), 7.42 (2 H, t, *J* 7.3, Fmoc Ar CH), 7.72 (2 H, d, *J* 7.3, Fmoc Ar CH), 7.89 (2 H, d, *J* 7.3, Fmoc Ar CH), 8.09 (1 H, t, *J* 6.0, FmocNH), 8.64 (1 H, br t, *J* 6.0, NH) and 12.49 (1 H, br s, CO_2H); δ_C(100.4 MHz; $DMSO_{d6}$)‡ 42.5 ($COCH_2CO$), 45.5 ($HNCH_2NH$), 46.7 (Fmoc CH), 65.8 (Fmoc CH_2), 120.2 (Fmoc Ar CH), 125.4 (Fmoc Ar CH), 127.2 (Fmoc Ar CH), 127.8 (Fmoc Ar CH), 140.8 (Fmoc Ar C), 143.9 (Fmoc Ar C), 156.4 (Fmoc CO), 166.1 (amide CO) and 169.4 (acid CO); m/z 355 [$(M+H)^+$, 355.1299, $C_{19}H_{19}N_2O_5$ requires 355.1294, 74%], 252 (13, $FmocNHCH_2^+$), 191 (13), 179 (97, 9-fluorenylmethyl cation), 178 (100, dibenzofulvene radical cation), 116 (22, $H_2CNHCOCH_2CO_2H^+$) and 94 (14).

(r) Solid Phase Synthesis of Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, ***146***

The SPS was performed under continuous flow conditions on a MilliGen 9050 PepSynthesizer. The synthesis was carried out on a 0.1 mmol scale, using a fourfold excess of reagents, on a PAL-PEG-PS[327] support (Millipore, 0.17 $mEqg^{-1}$, 590 mg). Freshly distilled DMF was used throughout. The resin was swollen in DMF before loading into the synthesizer. The standard HOBt solution [HOBt hydrate (5 g), acid violet 17 (25 mg) and DIPEA (67 μL) per 100 mL of DMF] was used as solvent during the coupling steps. The Fmoc deprotection steps were achieved with 20% piperidine in DMF and were carried out automatically, using the shortened "diketopiperidine deprotection protocol" for Fmoc-Glyψ(NHCO)Gly- residues and monitoring the deprotection profiles with the post-column detector (360 nm). The coupling steps which incorporated Fmoc-

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† The crude material's ^1H NMR spectrum also contained the following signals, due to impurities: 1.92 (0.1 H, s, acetic acid Me), 2.30 (0.4 H, s, toluene Me), 2.60 (0.3 H, s, HOSu CH_2), and 7.12-7.28 (0.4 H, m, toluene Ar CHs).

‡ The crude material's ^{13}C NMR spectrum also contained the following signals, due to impurities: 21.1 (toluene Me), 25.3 (HOSu CH_2), 128.3 (toluene Ar CH), 129.0 (toluene Ar CH) and 172.9 (HOSu CO).

Glyψ(NHCO)Gly, **143** [four aliquots of (144 mg, *ca.* 0.40 mmol§)], used DIPCDI [four aliquots of (63 μL, 50 mg, 0.40 mmol)] as the coupling reagent, added manually to 0.2 M Fmoc-Glyψ(NHCO)Gly, **143**, in the HOBt solution.¶ These coupling steps were prolonged manually until the pre-column detector reading (546 nm) reached a steady maximum. Gly^6 and Pro^5 were incorporated as their Fmoc-Xaa-OPfp derivatives (186 mg and 202 mg respectively, 0.40 mmol) at 0.3 M in the HOBt solution, under automatic control. *N*-Terminal capping with acetic anhydride (41 mg, 0.40 mmol) at 0.3 M in the HOBt solution, was performed automatically. Thus the coupling times were: Gly^9ψ(NHCO)Gly^{10}, 22 h; Gly^7ψ(NHCO)Gly^8, 24 h; Gly^6, 3 h; Pro^5, 1 h; Gly^3ψ(NHCO)Gly^4, 24 h; Gly^1ψ(NHCO)Gly^2, 48 h; and Ac, 40 m. After the assembly, the resin was removed from the synthesizer and washed successively with 20 mL aliquots of dichloromethane, methanol and ether, then dried under a stream of nitrogen.

The product, Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146**, was cleaved from the resin by standing in TFA / water (95:5, 15 mL) for 2 h. The resin was removed by filtration and washed with TFA (25 mL). The filtrate was evaporated, water (20 mL) added to the resultant green residue and the suspension freeze-dried to yield crude Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146**, as a pale green solid (50 mg, 75%); m/z (electrospray) 692.9 [$(M+Na)^+$, $C_{25}H_{39}N_{11}O_{11}Na$ requires 692.3, 100%], 670.7 [41, $(M+H)^+$, $C_{25}H_{40}N_{11}O_{11}$ requires 670.3], 641.3 (34), 578.3 (38), 555.7 (32), 514.0 (44), 486.3 (32), 442.1 {27, [Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-NH_2+H]$^+$}, 400.1 (34), 355.4 (35), 299.4 (22) and 278.4 (34).

A portion of the crude PMRI decapeptide, **146** (40 mg), was purified by successively washing with 10 mL aliquots of: solvent A plus B (50:50); water; ethanol; and ether. The respective solvent was added to the crude pseudopeptide, the suspension

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§ Corrected for HOSu content, as judged by ^{1}H NMR. Before use Fmoc-Glyψ(NHCO)Gly, **143** was dried under high vacuum over sodium hydroxide to remove all traces of acetic acid.

¶ Prior to embarking on this SPS a solubility test on the monomer, Fmoc-Glyψ(NHCO)Gly, **143**, indicated that it was necessary to perform its coupling steps at 0.2 M rather than the usual 0.3 M.

centrifuged (3000 rpm, 2 m) and the solvent decanted off. The resultant precipitate was dried under high vacuum to yield Ac-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-NH_2, **146**, as an off-white solid (8.7 mg); δ_H(400 MHz, $DMSO_{d6}$)* 1.74-2.18 (4 H, m, $Pro^5C^\beta H_2$ and $C^\gamma H_2$), 1.80 (3 H, s, Ac), 3.00-3.30 (12 H, m, $Gly^2C^\alpha H_2$, $Gly^4C^\alpha H_2$, $Gly^8C^\alpha H_2$ and $Gly^{10}C^\alpha H_2$)†, 3.45-3.58 (2 H, m, $Pro^5C^\delta H_2$), 3.58-3.73 (2 H, m, $Gly^6C^\alpha H_2$), 4.24-4.52 (9 H, m, $Gly^1C^\alpha H_2$, $Gly^3C^\alpha H_2$, $Gly^7C^\alpha H_2$, $Gly^9C^\alpha H_2$ and $Pro^5C^\alpha H$)‡, 7.05 (1 H, br s, NH_2), 7.41 (1 H, br s, NH_2), 8.16 (0.8 H, t, *J* 5.5, NH major conformer), 8.27 (0.3 H, t, *J* 6.1, NH minor conformer), 8.32 (0.8 H, t, *J* 5.9, NH major conformer), 8.48-8.64 (6.6 H, m, NHs) and 8.74 (0.6 H, t, *J* 5.3, NH major conformer).

3.2.3 *Val series.*

*(a) Preparation of Boc-Valψ(NHCO)Gly-OEt, **116(a)***[157]

Boc-valine **113(a)** (1.1 g, 5.0 mmol) was dissolved in THF (10 mL) and cooled to 0°C in an inert atmosphere. Tri-*n*-butyl amine (0.93 g, 1.2 mL, 5.0 mmol) followed by ethyl chloroformate (0.56 g, 0.49 mL, 5.1 mmol) were added slowly, with stirring and the temperature maintained at 0°C. Stirring was continued at 0°C for 30 m. An ice cold solution of sodium azide (0.66 g, 10.2 mmol) in the minimum volume of water (2.2 mL) was added and stirring continued for 1 h at 0°C. Ice cold portions of ethyl acetate (50 mL) and sat. sodium hydrogen carbonate (50 mL) were added and the mixture shaken and separated. The organic portion was washed with ice cold brine (50 mL), dried over

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* The ^1H NMR spectrum also contained the following signals due to impurities: 0.84-0.90 (2 H, m), 1.03-1.11 [3 H, two overlapping t, 1.05 (*J* 7.1, ethanol) and 1.09 (*J* 7.2, ether)], 1.21-1.39 (3 H, m), 4.11-4.15 (m), 7.65-7.73 (0.6 H, m) and 8.64-8.70 (0.6 H, m). The ^1H NMR spectrum of the crude material before washing was identical except for the presence of δ_H 3.50 (7 H,s) and the absense of δ_H 1.03-1.11 (m).

† It is unclear which (if any) of the signals in this region are due to impurities, nor how much the water signal contributes to the integral.

‡ This signal consisted of δ_H 4.26 (dd, *J* 2.9 and 8.3, $Pro^5C^\alpha H$ major conformer) and 4.33-4.52 (m).

magnesium sulfate and evaporated at <25°C.§ The resultant yellow oil, which effervesced slowly at r.t., was dissolved in toluene (10 mL): IR analysis at this stage indicated a mixture of acyl azide, **114(a)**, [ν_{max}(tol) 2140 cm^{-1}] and isocyanate, **115(a)**, [ν_{max}(tol) 2250 cm^{-1}]. The solution was heated to 70°C in an inert atmosphere, with stirring, for *ca.* 15 m (during which vigorous effervescence occurred), until IR analysis indicated all the acyl azide, **114(a)**, [ν_{max}(tol) 2140 cm^{-1}] had converted to the isocyanate, **115(a)**, [ν_{max}(tol) 2250 cm^{-1}]. The solution was maintained at 70°C and monoethyl malonate, **98** (0.80 g, 6.1 mmol) added. Stirring was continued at 70°C for *ca.* 5 m (during which further effervescence occurred), after which time IR analysis indicated all the isocyanate, **115(a)**, was consumed. The solution was cooled to r.t. and petrol (boiling range 40-60°C, 50 mL) was added. The resultant colourless precipitate was collected and dried under high vacuum. Column chromatography (sample preabsorbed from ethyl acetate solution) with ethyl acetate / petrol (50:50) as eluent, followed by recrystallisation from toluene / petrol yielded Boc-Valψ(NHCO)Gly-OEt, **116(a)**, as colourless needles (0.72 g, 48%)¶, m.p. 144-146°C (Found: C, 55.8; H, 8.85; N, 9.35. $C_{14}H_{26}N_2O_5$ requires C, 55.6; H, 8.65; N, 9.25%); $[\alpha]_D^{21}$ + 8.0 (*c* 1.05 in ethyl acetate); ν_{max} /cm^{-1} 3295s (NH), 1740s (ester carbonyl), 1685vs (Boc carbonyl), 1655vs (amide carbonyl), 1560m (amide II), 1510vs (amide II), 1410w, 1390w, 1300w, 1285w, 1250m, 1215w, 1175m, 1145s, 1125w, 1100w, 1080w, 1040m and 1020m; δ_H(270 MHz; $DMSO_{d6}$; TMS) 0.84 [3 H, d, *J* 6.8, CH(C*H*$_3$)$_2$], 0.85 [3 H, br d, *J* 6.6, CH(C*H*$_3$)$_2$], 1.17 (3 H, t, *J* 7.1, OCH_2CH_3), 1.38 (9 H, s, Boc), 1.81 (1 H, br m, C*H*$(CH_3)_2$], 3.19 (1 H, d, *J* 15.0, $COCH_2CO$), 3.27 (1 H, d, *J* 15.2, $COCH_2CO$), 4.06 (2 H, q, *J* 7.1, OCH_2CH_3), 4.94 [1 H, q, *J* 8.3, HN(C*H*Pr^i)NH], 7.08 (1 H, br d, *J* 7.5, BocNH) and 8.09 (1 H, d, *J* 8.4, NH); δ_C(67.8 MHz; $CDCl_3$; TMS)

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§ When the flask was removed from the rotary evaporator, air was readmitted *via* a calcium chloride / silica gel drying tube.

¶ The column chromatography also yielded a gummy colourless solid with ^{1}H NMR consistent with crude *N,N'*-bis[(*S*)-1-(*N*-Boc-amino)-2-methylpropyl]-urea, **117(a)**. This material was not further purified; δ_H (400 MHz; $DMSO_{d6}$; TMS) 0.81-0.88 [14 H, m, CH(C*H*$_3$)$_2$ plus impurity], 1.24-1.34 (11 H, impurity), 1.37 (10 H, s, Boc plus impurity), 1.80 (1 H, br s, C*H*$(CH_3)_2$], 2.32 (4 H, t, *J* 7.1, impurity), 4.77 [1 H, q, *J* 7.8, HN(C*H*Pr^i)NH], 6.19 (1 H, br s, BocNH) and 6.98 (1 H, br s, NH).

14.1 (OCH_2CH_3), 18.5 [$CH(CH_3)_2$], 28.3 [$(CH_3)_3C$], 31.8 [$CH(CH_3)_2$], 41.6 ($COCH_2CO$), 61.6 (OCH_2CH_3), 63.8 [$HN(CHPr^i)NH$], 79.9 [$(CH_3)_3C$], 155.1 (Boc CO), 164.8 (CO) and 169.2 (CO); m/z (C.I.) 303 [$(M+H)^+$, 4%], 259 (9), 247 [30, (M+H) - CH_2CMe_2], 203 (26, 247 - CO_2), 186 (100, 203 - NH_3), 172 (19), 159 (17), 132 [38, (M+H) - BocNCHPr^i], 116 (18) and 72 (45, $Pr^iCHNH_2^+$).

(b) Exhaustive tert-*Butoxycarbonylation of Boc-Valψ(NHCO)Gly-OEt,* ***116(a)***[312]

DMAP (46 mg, 0.37 mmol) and di-*tert*-butyl dicarbonate (81 mg, 0.37 mmol) were added to a solution of Boc-Valψ(NHCO)Gly-OEt, **116(a)** (51 mg, 0.17 mmol) in acetonitrile (1.0 mL) and the mixture stirred at r.t., in a dry atmosphere, for 22 h. After this time TLC (mixture C) indicated the presence of unreacted starting material, **116(a)**, therefore further di-*tert*-butyl dicarbonate (30 mg, 0.14 mmol) was added. Stirring was continued for an additional 24 h, after which time TLC indicated further, but incomplete, consumption of starting material, **116(a)**. The yellow reaction mixture was evaporated, the resultant yellow solid dissolved in ethyl acetate (50 mL) and the solution successively washed with 1 M potassium hydrogen sulfate (2 x 20 mL) and brine (20 mL). The organic layer was dried over sodium sulfate and evaporated. Column chromatography of the resultant yellow solid (preabsorbed from ethyl acetate solution), with ethyl acetate / petrol (30:70) as eluent, yielded:

ethyl *N*-[(*S*)-1-(*N'*-Boc-amino)-2-methylpropyl]-(*R,S*)-2-Boc-malonamate, **128**, as a colourless solid (38 mg, 56%) [which slowly reverted to starting material (as shown by TLC) during analysis (the order of the analysis, and thus the order of increasing impurity, was: ^{1}H NMR, ^{13}C NMR and m.s.)]; δ_H(270 MHz; $CDCl_3$; TMS) 0.96 [6 H, d, *J* 6.6, $CH(CH_3)_2$], 0.97 [6 H, d, *J* 6.8, $CH(CH_3)_2$], 1.29 (3 H, t, *J* 7.1, OCH_2CH_3), 1.30 (3 H, t, *J* 7.1, OCH_2CH_3), 1.44 (18 H, s, NBoc), 1.47 (9 H, s, Boc), 1.48 (9 H, s, Boc), 1.90 (1.2 H, br s, impurity or $CH(CH_3)_2$ minor conformer], 2.22 (1.4 H, br s, $CH(CH_3)_2$], 4.21 [2 H, s, COCH(Boc)CO], 4.24 (2 H, q, *J* 7.1, OCH_2CH_3), 4.25 (2 H, m, OCH_2CH_3)*, 4.92 [2 H, br s, HN(CHPr^i)NH], 5.33 (2 H, br s, BocNH) and 7.76 (2 H, br s, NH); δ_C(100.4 MHz;

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* This signal consisted of two closely overlapping quartets, *J* 7.1.

$CDCl_3$)† 13.9 (OCH_2CH_3), 18.4 [$CH(CH_3)_2$], 18.5 [$CH(CH_3)_2$], 27.7 [$(CH_3)_3C$], 28.2 [$(CH_3)_3C$], 31.7 [$CH(CH_3)_2$], 31.9 [$CH(CH_3)_2$], 60.3 [COCH(Boc)CO], 62.3 (OCH_2CH_3), 63.9 [br, HN(CHPr^i)NH], 79.7 [br, $(CH_3)_3COCONH$], 83.7 [$(CH_3)_3COCOCH$], 83.8 [$(CH_3)_3COCOCH$], 154.9 (NBoc CO), 155.0 (NBoc CO), 162.2 (br, Boc CO), 164.4 (CO), 164.6 (CO), 165.6 (CO) and 165.8 (CO); m/z 425 [$(M+Na)^+$, 16%), 403 [23, $(M+H)^+$], 347 [7, (M+H) - CH_2CMe_2], 303 {9, 347 - CO_2 and / or [**116(a)**+H]$^+$]} 286 (21, 347 - H_2O, CO, NH), 247 (14, 303 - CH_2CMe_2), 230 (51, 286 - CH_2CMe_2), 186 (33, 230 - CO_2), 176 (16), 132 [14, $H_2NC(OH)CH_2CO_2Et^+$], 116 (26) and 72 (100, Pr^i$CHNH_2^+$);

and Boc-Valψ(NHCO)Gly-OEt, **116(a)** (4.4 mg, 9%); confirmed by TLC.

(c) N-*Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt,* ***116(a)****, with TFA*

(i) In the presence of triethylsilane[243]

TFA (30 mg, 0.2 mL, 2.6 mmol) and triethylsilane (55 mg, 76 μL, 0.48 mmol) were added to a solution of Boc-Valψ(NHCO)Gly-OEt, **116(a)** (58 mg, 0.19 mmol) in dichloromethane (0.3 mL). The mixture was stirred at r.t. in a dry atmosphere for 2 h, after which time TLC (mixture C) indicated all the starting material, **116(a)**, was consumed. The mixture was evaporated to dryness and the residue triturated with ether. The ether was decanted off and the colourless residue collected by dissolving in ethanol, evaporation and drying under high vacuum. Evaporation of the ether and additional trituration / decantation / evaporation cycles yielded further product.‡ The resultant colourless oil was dissolved in water and freeze dried to yield crude TFA.Valψ(NHCO)Gly-OEt, **119**, as a hygroscopic, cloudy, colourless gum (50 mg, 84%); $[\alpha]_D^{18}$ + 12.1 (*c* 0.57 in ethanol); ν_{max} /cm^{-1} 3420s (NH), 3200s (NH), 3050s (NH_3^+), 1735vs (ester carbonyl), 1675br,vs (carboxylate and amide carbonyl), 1535s (amide II), 1430s, 1325m, 1200vs (C-F and / or C-O), 1135vs (C-F and / C-O), 1030s, 1000m, 835m, 800m, 765m and 720m (C-F);

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† The ^{13}C NMR spectrum also contained the following signals, due to impurities: 14.0 [**116(a)** OCH_2CH_3], 31.2 [$CH(CH_3)_2$], 41.5 [**116 (a)** $COCH_2CO$] and 61.5 [**116 (a)**OCH_2CH_3].

‡ The total number of such cycles necessary to achieve good recovery of the product varied between 1 and 5 from experiment to experiment.

δ_H(270 MHz; $DMSO_{d6}$)‡ 0.94 [6 H, d, *J* 7.0, $CH(CH_3)_2$], 1.19 (3 H, t, *J* 7.2, OCH_2CH_3), 1.99 (1 H, octet, *J* 7.0, $CH(CH_3)_2$], 3.30 (1 H, d, *J* 15.4, $COCH_2CO$), 3.38 (1 H, d, *J* 15.2, $COCH_2CO$), 4.09 (2 H, q, *J* 7.1, OCH_2CH_3), 4.70 [1 H, t, *J* 8.1, $HN(CHPr^i)NH$], 8.26 (3 H, br s, ^+H_3N) and 8.90 (1 H, d, *J* 8.4, NH); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 17.0 [$CH(CH_3)_2$], 18.1 [$CH(CH_3)_2$], 30.4 [$CH(CH_3)_2$], 42.3 ($COCH_2CO$), 60.7 (OCH_2CH_3), 61.6 [$HN(CHPr^i)NH$], 158.4 (q, *J* 31, $CF_3CO_2^-$), 166.4 (CO) and 167.4 (CO); m/z 405 [$(2M-H)^+$, 23%], 257 (12), 203 (33, M^+, 203.1384, $C_9H_{19}N_2O_3$ requires 203.1396), 186 (100, M - NH_3) and 72 (87, $Pr^iCHNH_2^+$); m/z (-ve) 341 [$(3CF_3CO_2+2H)^-$, 16%], 287 (14), 227 [100, $(2CF_3CO_2+H)^-$] and 113 (71, $CF_3CO_2^-$).

(ii) In the absence of triethylsilane

TFA (1.2 g, 0.8 mL, 10 mmol) was added to a solution of Boc-Valψ(NHCO)Gly-OEt, **116(a)** (237 mg, 0.78 mmol) in dichloromethane (1.3 mL). The mixture was stirred at r.t. in a dry atmosphere for 135 m, after which time TLC (mixture C) indicated all the starting material, **116(a)**, was consumed. The mixture was evaporated to dryness, petrol (8 mL) added and the mixture slowly stirred for a few hours.[263] The petrol was decanted off and the process repeated with a further aliquot of petrol (8 mL). The resultant residue was triturated with ether. The ether was decanted off and the colourless residue collected by dissolving in ethanol, evaporation and drying under high vacuum. Evaporation of the ether and additional trituration / decantation / evaporation cycles yielded further product.§ Thus crude TFA.Valψ(NHCO)Gly-OEt, **119**, was obtained as a hygroscopic, cloudy, colourless gum (207 mg, 84%), identical to the above.

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‡ The crude material's ^1H NMR spectrum also contained the following signals, due to impurities: δ_H 0.84 (0.4 H, d *J* 6.6), 0.90 (0.1 H, s), 2.88 (0.1 H, t, *J* 6.0), 3.18 (0.3 H, d, *J* 10.3) and 7.18 (0.8 H, t, $J_{H,N}$ 51.8, NH_4^+).

§ The total number of such cycles necessary to achieve good recovery of the prodcut varied between 3 and 5 from experiment to experiment.

(d) N-*Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt,* ***116(a)****, with ethanolic hydrogen chloride*

Boc-Valψ(NHCO)Gly-OEt, **116(a)** (200 mg, 0.66 mmol) was dissolved in 5 M HCl in ethanol (3.3 mL) and stirred at r.t., in a dry atmosphere for 3 h. The solution was diluted with ethanol, evaporated and dried under high vacuum to provide a yellow residue. ^{1}H NMR ($DMSO_{d6}$) and TLC (mixture A) revealed that this residue was a mixture of compounds, probably including the desired HCl.Valψ(NHCO)Gly-OEt. In order to identify the impurities, the mixture was column chromatographed (sample applied as a slurry in the initial eluent) with ethyl acetate / petrol (50:50 then 100:0), as eluent. The pooled fractions yielded a colourless solid (23 mg), which was subsequently revealed to be a mixture of ethyl malonamate, **118**, and ethylmalonyl-Valψ(NHCO)Gly-OEt, **120**, in a molar ratio of 63:37; R_F 0.4 (ethyl acetate), 0.4 and 0.5 (mixture E) and 0.1 (ether); δ_H(270 MHz, $DMSO_{d6}$) 0.86 [6 H, d, *J* 6.6, $CH(CH_3)_2$], 1.18 (11.4 H, m, OCH_2CH_3)¶, 1.91 [1 H, octet, *J* 7.0, $CH(CH_3)_2$], 3.17 (3.2 H, s, $COCH_2CO$), 3.18 (2 H, d, *J* 15.4, $COCH_2CO$), 3.28 (2.6 H*, d, *J* 15.0, $COCH_2CO$), 4.06 (7.5 H, m, OCH_2CH_3)†, 5.17 [1 H, q, *J* 8.1, $HN(CHPr^i)NH$], 7.10 (1.5 H, br s, NH_2), 7.52 (1.5 H, br s, NH_2) and 8.26 (2 H, d, *J* 8.4, NH); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 18.2 [$CH(CH_3)_2$], 31.7 [$CH(CH_3)_2$], 42.5 ($COCH_2CO$), 60.4 [$HN(CHPr^i)NH$ and OCH_2CH_3], 60.5 (OCH_2CH_3), 164.6 (CO), 167.3 (CO), 167.9 (CO) and 168.0 (CO); m/z 317 [(**120**+H)$^+$, 36%], 186 (100, $Pr^iCHNHCOCH_2CO_2Et^+$) and 132 [46, (**118**+H)$^+$].

(e) Coupling of monoethyl malonate, ***98****, and ammonia*

N-Hydroxysuccinimide (453 mg, 3.9 mmol) and EDC.HCl (754 mg, 3.9 mmol) were added to a solution of monoethyl malonate, **98** (520 mg, 3.9 mmol) in acetonitrile (5 mL) and the mixture stirred in a dry atmosphere, at r.t. for 75 m. Aq. ammonia (*ca.* 33% w/w, 0.23 mL, 3.9 mmol NH_3) was added to the reaction mixture, and the cloudy solution

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¶ This signal consisted of two overlapping triplets: δ_H 1.17 (t, *J* 7.0) and 1.18 (t, *J* 7.1).

* Due to overlap with the water signal.

† This signal consisted of two overlapping quartets: δ_H 4.06 (q, *J* 7.0) and 4.06 (t, *J* 7.0).

stirred at r.t. overnight. After this time, TLC (mixture E) indicated incomplete consumption of the monoethyl malonate, **98**, so further EDC.HCl (377 mg, 2.0 mmol) was added and stirring continued. After an additional 6 h, unreacted monoethyl malonate, **98**, still remained (as indicated by TLC), so further EDC.HCl (377 mg, 2.0 mmol) was added and stirring continued overnight. TLC then indicated complete consumption of the monoethyl malonate, **98**, so the solution was concentrated *in vacuo* and dichloromethane (70 mL) added to the residual solution. The mixture was washed with brine (15 mL), and the brine back-extracted with dichloromethane (70 mL). The combined organic extracts were dried over sodium sulfate, evaporated and dried under high vacuum. Column chromatography (sample preabsorbed from ethyl acetate solution), with ethyl acetate as the eluent, yielded ethyl malonamate, **118**, as a colourless oil, which crystallised as colourless needles on standing in the refridgerator (306 mg, 59%); m.p. 39-42°C (from ether) (lit.,[262] 42°C) (Found: C, 45.6; H, 7.0; N, 10.6. Calculated for $C_5H_9NO_3$: C, 45.8; H, 6.9; N, 10.7); R_F 0.4 (ethyl acetate), 0.4 (mixture E) and 0.1 (ether); ν_{max} / cm^{-1} 3390s (NH), 3325m (NH), 3310s (NH), 1720br,vs (ester carbonyl), 1665br,vs (amide carbonyl), 1625s (amide II), 1425s, 1315s, 1285s, 1170s, 1130m, 1115m, 1030s, 965w, 880m and 850m; δ_H(270 MHz, $DMSO_{d6}$; TMS) 1.18 (3 H, t, *J* 7.1, OCH_2CH_3), 3.18 (2 H, s, $COCH_2CO$), 4.07 (2 H, q, *J* 7.1, OCH_2CH_3), 7.10 (1 H, br s, NH_2) and 7.52 (1 H, br s, NH_2); δ_C(67.8 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 42.5 ($COCH_2CO$), 60.4 (OCH_2CH_3), 167.3 (CO) and 168.0 (CO); m/z (C.I.) 132 [$(M+H)^+$, 100%], 115 [5, (M+H) - NH_3], 104 [3, (M+H) - C_2H_4] and 86 (4, 104 - H_2O).

*(f) Coupling of monoethyl malonate, **98**, and TFA.Valψ(NHCO)Gly-OEt, **119***

Monoethyl malonate, **98** (108 mg, 0.82 mmol), crude TFA.Valψ(NHCO)Gly-OEt, **119** (258 mg, *ca.* 0.82 mmol) and *N*-hydroxysuccinimide (94 mg, 0.82 mmol) in THF (10 mL) were cooled to -10°C, in a dry atmosphere. EDC.HCl (170 mg, 0.89 mmol) and triethylamine (227 μL, 165 mg, 1.6 mmol) were added and the mixture stirred in a dry atmosphere, at -10°C for 4 h, and then at r.t. for 2 days. The solution was evaporated to dryness. The resultant off-white residue was dissolved in dichloromethane (100 mL), the solution washed with 5% citric acid (50 mL) and separated. The aq. layer was back-

extracted with dichloromethane (50 mL), the combined organic extracts were washed with water (50 mL plus a few drops of brine), dried over sodium sulfate, evaporated and dried under high vacuum. Column chromatography (sample applied as a solution in the eluent), with chloroform / methanol / acetic acid (95:3:2) as the eluent, yielded ethylmalonyl-Valψ(NHCO)Gly-OEt [bis-(*N*-ethylmalonyl)-1,1-diamino-2-methylpropane], **120**, as a colourless solid. Crystallisation from ethyl acetate yielded ethylmalonyl-Valψ(NHCO)Gly-OEt, **120**, as colourless needles (73 mg, 28%); m.p. 150-151°C (change in appearance noted at 99-105°C and 139-143°C) (Found: C, 53.1; H, 7.6; N, 8.85. $C_{14}H_{24}N_2O_6$ requires C, 53.15; H, 7.65; N, 8.85); R_F 0.4 (ethyl acetate), 0.5 (mixture E) and 0.1 (ether); ν_{max} / cm^{-1} 3255s (NH), 3140m (NH), 3050w (NH), 1740s (ester carbonyl), 1730s (ester carbonyl), 1660vs (amide carbonyl), 1585w (amide II), 1540m (amide II), 1420w, 1330s, 1205s, 1180m, 1130w, 1080m, 1035w, 865w, 800w, 725w and 625w; δ_H(270 MHz, $DMSO_{d6}$; TMS) 0.87 [6 H, d, *J* 6.6, $CH(CH_3)_2$], 1.17 (6 H, t, *J* 7.1, OCH_2CH_3), 1.90 [1 H, octet, *J* 7.1, $CH(CH_3)_2$], 3.19 (2 H, d, *J* 15.2, $COCH_2CO$), 3.28 (2 H, d, *J* 15.0, $COCH_2CO$), 4.06 (4 H, q, *J* 7.1, OCH_2CH_3), 5.17 [1 H, q, *J* 8.2, $HN(CHPr^i)NH$] and 8.27 (2 H, d, *J* 8.4, NH); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 18.2 [$CH(CH_3)_2$], 31.7 [$CH(CH_3)_2$], 42.5 ($COCH_2CO$), 60.4 [$HN(CHPr^i)NH$], 60.5 (OCH_2CH_3), 164.6 (CO) and 167.9 (CO); m/z (FAB, glycerol) 317 [$(M+H)^+$, 317.1716, $C_{14}H_{25}N_2O_6$ requires 317.1713, 63%], 172 (15), 158 (15), 140 (15) and 72 (100, $Pr^iCHNH_2^+$).

*(g) C-Terminal deprotection of Boc-Valψ(NHCO)Gly-OEt, **116(a)**, by saponification*

2 M Sodium hydroxide (14 mL) was added to a solution of Boc-Valψ(NHCO)Gly-OEt, **116(a)** (3.6 g, 12 mmol) in ethanol (65 mL). The mixture was stirred at r.t. overnight and acidified to pH 2/3 by careful addition of 1 M potassium hydrogen sulfate. Brine (250 mL) was added and the solution extracted with ethyl acetate (5 x 250 mL). The organic extract was dried over sodium sulfate, evaporated and dried under high vacuum to yield Boc-Valψ(NHCO)Gly, **121**, as a colourless solid (3.3 g, 99%), m.p. 118-121°C (Found: C, 52.5; H, 8.1; N, 10.0. $C_{12}H_{22}N_2O_5$ requires C, 52.55; H, 8.1; N, 10.2%); $[\alpha]_D^{19}$ + 12.0 (*c* 1.16 in ethanol); ν_{max} /cm^{-1} 3325s (NH), 3100-2500br,w (CO_2H), 1725s

(acid carbonyl), 1690vs (Boc carbonyl), 1655m (amide carbonyl), 1560m (amide II), 1515s (amide II), 1315w, 1290w, 1250w, 1215w, 1175m, 1150m, 1070w, 1045w and 1025w; δ_H(270 MHz, $DMSO_{d6}$) 0.83 [3 H, d, *J* 6.6, $CH(CH_3)_2$], 0.84 [3 H, br d, *J* 6.6, $CH(CH_3)_2$], 1.37 (9 H, s, Boc), 1.81 (1 H, m, $CH(CH_3)_2$], 3.11 (1 H, d, *J* 15.4, $COCH_2CO$), 3.18 (1 H, d, *J* 15.4, $COCH_2CO$), 4.94 [1 H, q, *J* 8.3, $HN(CHPr^i)NH$], 7.05 (1 H, d, *J* 7.7, BocNH), 8.05 (1 H, d, *J* 8.4, NH) and 12.47 (1 H, br s, CO_2H); δ_C(67.8 MHz; $DMSO_{d6}$) 18.2 [$CH(CH_3)_2$], 18.3 [$CH(CH_3)_2$], 28.2 [$(CH_3)_3C$], 32.0 [$CH(CH_3)_2$], 42.5 ($COCH_2CO$), 61.9 [$HN(CHPr^i)NH$], 78.2 [$(CH_3)_3C$], 154.6 (Boc CO), 164.9 (amide CO) and 169.5 (acid CO); m/z 275 [$(M+H)^+$, 56%), 219 [59, (M+H) - CH_2CMe_2], 158 (100, 219 - H_2O, CO, NH), 116 [30, $CH_2NHCOCH_2CO_2H^+$], 104 [23, (M+H) - $BocNCHPr^i$] and 89 (14).

(h) Coupling of Boc-Valψ(NHCO)Gly, ***121****, and TFA.Valψ(NHCO)Gly-OEt,* ***119***

A solution of crude TFA.Valψ(NHCO)Gly-OEt, **119** (125.6 mg, *ca.* 0.40 mmol), Boc-Valψ(NHCO)Gly, **121** (109 mg, 0.40 mmol), EDC.HCl (77 mg, 0.40 mmol) and *N*-hydroxysuccinimide (46 mg, 0.40 mmol) in THF (2.5 mL) was cooled to -10°C in a dry atmosphere and triethylamine (0.11 mL, 80 mg, 0.79 mmol) added. The mixture was stirred at -10°C for 4 h, and then at r.t. for a further 2 days. The solution was evaporated to dryness, the resultant residue washed with water, the colourless precipitate collected and dried under high vacuum over phosphorus pentoxide. Column chromatography of the precipitate (applied as a slurry in the eluent), with chloroform / methanol (95:5) as eluent, yielded crude Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**. This material was further purified by dissolving in hot ethanol and collecting the colourless solid which precipitated on cooling, to yield Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, as a colourless solid (49 mg, 27%); m.p. 224-227°C; $[\alpha]_D^{19}$ + 3.8 (*c* 0.21 in DMSO); ν_{max} / cm^{-1} 3295s (NH), 3260s (NH), 3110w (NH), 1745s (ester carbonyl), 1715m (ester carbonyl), 1695s (Boc carbonyl), 1670br,vs (amide carbonyl), 1650s (amide carbonyl), 1565s (amide II), 1555s (amide II), 1520s (amide II), 1390m, 1310m, 1255m, 1175m, 1160m, 1145s, 1075m, 1045m, 1025m, 960m, 880w and 725m; δ_H(270 MHz, $DMSO_{d6}$, TMS) 0.80-0.87 [12 H, m, $CH(CH_3)_2$], 1.17 (3 H, t, *J* 7.2, OCH_2CH_3), 1.37 (9 H, s, Boc), 1.71-1.94 [2 H,

m, C*H*(CH_3)$_2$], 3.06 (1 H, d, *J* 15.0, Gly^{2}C$^\alpha$H$_2$), 3.11 (1 H, d, *J* 15.0, Gly^{2}C$^\alpha$H$_2$), 3.17 (1 H, d, *J* 15.0, Gly^{4}C$^\alpha$H$_2$), 3.27 (1 H, d, *J* 15.0, Gly^{4}C$^\alpha$H$_2$), 4.06 (2 H, q, *J* 7.1, OC*H*$_2$CH$_3$), 4.97 [1 H q, *J* 8.4, Val^{1}C$^\alpha$H], 5.22 [1 H q, *J* 8.1, Val^{3}C$^\alpha$H], 7.07 (1 H, br d, *J* 9.0, BocNH), 8.04 (1 H, d, *J* 8.4, Val$^1\psi$NH), 8.23 (1 H, d, *J* 8.1, Val^{3}NH) and 8.28 (1 H, d, *J* 8.5, Val$^3\psi$NH); δ_C(100.4 MHz; DMSO$_{d6}$) 14.1 (OCH$_2$*C*H$_3$), 18.1 [CH(*C*H$_3$)$_2$], 18.1 [CH(*C*H$_3$)$_2$], 18.3 [CH(*C*H$_3$)$_2$], 18.3 [CH(*C*H$_3$)$_2$], 28.3 [(*C*H$_3$)$_3$C], 31.8 [*C*H(CH$_3$)$_2$], 32.2 [*C*H(CH$_3$)$_2$], 42.5 (CO*C*H$_2$CO), 42.8 (CO*C*H$_2$CO), 60.1 (O*C*H$_2$CH$_3$), 60.5 [HN(*C*HPr^i)NH], 61.7 [HN(*C*HPr^i)NH], 77.9 [(CH$_3$)$_3$*C*], 154.7 (Boc CO), 164.5 (CO), 166.0 (CO), 166.3 (CO) and 167.9 (CO); m/z 459 [(M+H)$^+$, 459.2808, $C_{21}H_{39}N_4O_7$ requires 459.2819, 14%), 342 [36, (M+H) - CH_2CMe_2, H_2O, CO, NH], 288 [24, (M+H) - BocNCHPr^i], 272 (7), 211 (38, Pr^{i}CHNHCOCH$_2$CONCHPr^{i+}), 186 (56, Pr^{i}CHNHCOCH$_2$CO$_2$Et$^+$), 157 (90, H$_2$NCOCH$_2$CONHCHPr^{i+}), 72 (100, Pr^{i}CHNH$_2^+$) and 57 (22, Bu^{t+}).

(i) Preparation of Boc-Valψ(NHCO)Gly-OSu, 123

(i) DCC Method[242]

A solution of Boc-Valψ(NHCO)Gly, **121** (0.99 g, 3.6 mmol) and *N*-hydroxysuccinimide (0.46 g, 4.0 mmol) in THF (20 mL) was cooled to -10°C. DCC (0.82 g, 4.0 mmol) was added and the mixture stirred in a dry atmosphere at -10°C for 4 h, and then at r.t. overnight. The precipitated DCU was filtered off and washed with ethyl acetate. The filtrate was evaporated and dried under high vacuum to yield crude Boc-Valψ(NHCO)Gly-OSu, **123**, as a colourless solid (1.2 g, 87%); δ_H(270 MHz, CDCl$_3$, TMS)‡ 0.87 [3 H, d, *J* 7.0, CH(C*H*$_3$)$_2$], 0.89 [3 H, d, *J* 7.0, CH(C*H*$_3$)$_2$], 1.37 (9 H, s, Boc), 2.11 (1 H, br s, C*H*(CH$_3$)$_2$], 2.81 (4 H, s, succinimide CH$_2$), 3.56 (2 H, s, COCH$_2$CO), 4.89 [1 H, br s, HN(C*H*Pr^i)NH], 5.22 (1 H, br s, BocNH) and 7.19 (1 H, br s, NH); m/z 743.3 [(2M+H)$^+$, 10%], 394.2 [12, (M+Na)$^+$], 372.2 [72, (M+H)$^+$], 316.2 [46, (M+H) - CH_2CMe_2], 255.1 (100, 316.2 - H_2O, CO, NH), 225.2 [39, (DCU+H)$^+$], 201.1 [12,

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‡ The crude material's ^{1}H NMR spectrum also contained the following signals, due to impurities: 0.90-1.32 (3 H, m, DCU H$_{ax}$), 1.42-2.00 (5.3 H, m, DCU H$_{eq}$ + unknown), 2.76 (0.5 H, s, HOSu CH$_2$) and 3.40 (0.5 H, m, DCU CH).

(M+H) - BocNCHPr^i], 172.1 (12), 116.1 [26, (HOSu+H)$^+$], 99.1 (13), 86.1 (16) and 72 (83, Pr^{i}CHNH$_2^+$).

(ii) DSC Method[264]

To a solution of Boc-Valψ(NHCO)Gly, **121** (109 mg, 0.40 mmol) in acetonitrile (2 mL), pyridine (32 μL, 31 mg, 0.40 mmol) and DSC (102 mg, 0.40 mmol) were successively added and the resultant suspension stirred in a dry atmosphere at r.t.. After 50 m the solution was yellow and homogeneous, and stirring was continued. After 4 days TLC (mixture E) indicated that some Boc-Valψ(NHCO)Gly, **121**, remained unreacted, therefore additional DSC (102 mg, 0.40 mmol) was added, and stirring continued. After a further 2 days TLC still indicated incomplete reaction, therefore additional DSC (102 mg, 0.40 mmol) was added, and stirring continued. After a further day, although TLC indicated traces of Boc-Valψ(NHCO)Gly, **121**, remaining, the reaction was worked-up as follows. The solution was evaporated, the resultant cloudy yellow residue dissolved in dichloromethane (50 mL) and washed successively with 5% citric acid (25 mL) and water (25 mL). The organic layer was dried over sodium sulfate, evaporated and dried under high vacuum to yield crude Boc-Valψ(NHCO)Gly-OSu, **123**, as a colourless solid [147 mg, quantitative; TLC (mixture E) of the crude material revealed the presence of traces of Boc-Valψ(NHCO)Gly, **121**], m.p. 154-159°C; [$\alpha]_D^{27}$ + 1.5 (*c* 0.20 in dichloromethane); ν_{max} /cm^{-1} 3340s (NH), 3315s (NH), 1820m (succinimide carbonyl), 1790m (succinimide carbonyl), 1740vs (ester carbonyl), 1690vs (Boc carbonyl), 1660m (amide carbonyl), 1655m (amide carbonyl), 1545m (amide II), 1505m (amide II), 1285s, 1245m, 1195s, 1165w, 1140m, 1085m, 1055m, 1020m, 1000m, 970w, 945m, 835m and 705vs; δ_H(270 MHz, $CDCl_3$, TMS)§ 0.86 [3 H, d, *J* 7.0, CH(C*H*$_3$)$_2$], 0.87 [3 H, d, *J* 6.6, CH(C*H*$_3$)$_2$], 1.35 (9 H, s, Boc), 2.09 (1 H, br s, C*H*(CH$_3$)$_2$], 2.80 (4 H, s, succinimide CH$_2$), 3.55 (2 H, s, COCH$_2$CO), 4.87 [1 H, br s, HN(C*H*Pr^i)NH], 5.26 (1 H, br s, BocNH) and 7.19 (1 H, br s, NH); m/z 372 [(M+H)$^+$, 372.1807, $C_{16}H_{26}N_3O_7$ requires 372.1771, 77%], 316 [45, (M+H) - CH_2CMe_2], 272 (12), 255 (100, 316 - H_2O, CO, NH), 201 [12, (M+H) -

§ The crude material's ^{1}H NMR spectrum also contained the following signals, due to impurities: 1.68-1.93 (0.6 H, br s), 2.64 (0.1 H, s) and 3.03 (0.4 H, s).

BocNCHPr^i], 172 (16), 163 (12), 147 (10), 141 (13), 116 [29, (HOSu+H)$^+$], 99 (19), 86 (22) and 72 (90, Pr^{i}CHNH$_2^+$).

*(j) Coupling of Boc-Valψ(NHCO)Gly-OSu, **123**, and TFA.Valψ(NHCO)Gly-OEt, **119***

A solution of crude Boc-Valψ(NHCO)Gly-OSu, **123** (1.17 g, *ca.* 3.1 mmol, synthesised by the DCC method) in THF (100 mL) was added by decantation (leaving behind residual DCU) to crude TFA.Valψ(NHCO)Gly-OEt, **119** (995 mg, *ca.* 3.1 mmol). Triethylamine (440 μL, 318 mg, 3.1 mmol) was added and the mixture stirred at r.t., in a dry atmosphere, for 3 days, after which time TLC (mixture E) indicated consumption of the Boc-Valψ(NHCO)Gly-OSu, **123**, was complete. The solution was evaporated to dryness, the resultant off-white solid washed with water and the colourless precipitate collected. The precipitate was dried under high vacuum over phosphorus pentoxide. Column chromatography of the precipitate (applied as a slurry in the eluent), with chloroform / methanol (95:5) as eluent, yielded crude Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**. This material was further purified by dissolving in hot ethanol and collecting the colourless solid which precipitated on cooling, to yield Boc-Valψ(NHCO)Gly-Valψ(NHCO)Gly-OEt, **122**, as a colourless solid [191 mg, 12% over the two steps from Boc-Valψ(NHCO)Gly, **121**], identical to the above.

*(k) Coupling of Boc-Valψ(NHCO)Gly, **121**, and HCl.Pro-Gly-OEt, **129***

A solution of Boc-Valψ(NHCO)Gly, **121** (529 mg, 1.9 mmol), HCl.Pro-Gly-OEt, **129** (503 mg, 2.1 mmol) and *N*-hydroxysuccinimide (396 mg, 1.9 mmol) in DMF (6 mL) was cooled to -10°C in a dry atmosphere. DCC (398 mg, 1.9 mmol) and triethylamine (270 μL, 195 mg, 1.9 mmol) were successively added at -10°C, with stirring. The mixture was stirred at -10°C for 4 h, and then at r.t. for a further 6 days. The precipitated DCU was filtered off and washed with dichloromethane (40 mL). The filtrate was evaporated to produce a cloudy yellow residue which was dissolved in dichloromethane (150 mL) and the solution successively washed with 5% citric acid (60 mL plus a few drops of brine) and water (50 mL). The aq. layers were back-extracted with dichloromethane (2 x 50 mL), the combined organic extracts dried over sodium sulfate and evaporated. Column chromatography of the resultant off-white solid (applied as a solution in the eluent), with

chloroform / methanol (95:5) as eluent, furnished crude Boc-Valψ(NHCO)Gly-Pro-Gly-OEt, **133**, which was crystallised from ethanol to yield pure Boc-Valψ(NHCO)Gly-Pro-Gly-OEt, **133**, as a colourless solid (196 mg, 22%); m.p. 160-162°C (Found: C, 55.25; H, 8.05; N, 12.25. $C_{21}H_{36}N_4O_7$ requires C, 55.25; H, 7.95; N, 12.25%); $[\alpha]_D^{21}$ - 47.8 (*c* 0.26 in ethanol); ν_{max} /cm^{-1} 3335s (NH), 3300vs (NH), 3080w (NH), 1730s (ester carbonyl), 1690vs (Boc carbonyl), 1660vs (amide carbonyl), 1650vs (amide carbonyl), 1645vs (amide carbonyl), 1555s (amide II), 1550s (amide II), 1515s (amide II), 1335s, 1290s, 1250s, 1230s, 1175s, 1150s, 1100w, 1075w, 1040m, 1020m, 965w, 915w, 875w, 855w, 780w and 720w; δ_H(270 MHz, DMSO$_{d6}$; TMS) 0.80-0.87 [6 H, br m, CH(C*H*$_3$)$_2$], 1.15-1.21 (3 H, m, OCH$_2$C*H*$_3$)[¶], 1.37 (9 H, s, Boc), 1.69-2.27 (5 H, m, C*H*(CH$_3$)$_2$, Pro^{3}C$^\beta$H$_2$ and C$^\gamma$H$_2$), 2.99-3.33 (2 H, m, Gly^{2}C$^\alpha$H$_2$), 3.37-3.63 (2 H, m, Pro^{3}C$^\delta$H$_2$), 3.75 (0.8 H, dd, *J* 6.0 and 17.4, Gly^{4}C$^\alpha$H$_2$ major conformer), 3.80-3.88 (1.2 H, m, Gly^{4}C$^\alpha$H$_2$ both conformers)[*], 4.04-4.12 (2 H, m, OC*H*$_2$CH$_3$)[†], 4.31 (0.7 H, br dd, *J* 1.8 and 7.5, Pro^{3}C$^\alpha$H major conformer), 4.55 (0.3 H, dd, *J* 2.7 and 8.9, Pro^{3}C$^\alpha$H minor conformer), 4.98 (1 H, q, *J* 8.3, Val^{1}C$^\alpha$H), 7.06 (1 H, br t, *J* 9.0, BocNH), 8.16 (1 H, t, *J* 9.2, Val$^1\psi$NH), 8.27 (0.7 H, t, *J* 5.9, Gly^{4}NH major conformer) and 8.56 (0.3 H, t, *J* 6.3, Gly^{4}NH minor conformer); δ_C(100.4 MHz; DMSO$_{d6}$; TMS) 13.9 (OCH$_2$*C*H$_3$), 18.0 [CH(*C*H$_3$)$_2$ minor conformer], 18.1 [CH(*C*H$_3$)$_2$], 18.2 [CH(*C*H$_3$)$_2$], 22.2 (Pro^{3}C$^\gamma$H$_2$ minor conformer), 23.7 (Pro^{3}C$^\gamma$H$_2$ major conformer), 28.1 [(*C*H$_3$)$_3$C], 29.5 (Pro^{3}C$^\beta$H$_2$ major conformer), 31.5 (Pro^{3}C$^\beta$H$_2$ minor conformer), 31.9 [*C*H(CH$_3$)$_2$ major conformer], 32.1 [*C*H(CH$_3$)$_2$ minor conformer], 40.5 (Gly^{4}C$^\alpha$H$_2$ major conformer), 40.7 (Gly^{4}C$^\alpha$H$_2$ minor conformer), 41.7 (Gly^{2}C$^\alpha$H$_2$ minor conformer), 42.2 (Gly^{2}C$^\alpha$H$_2$ major conformer), 46.3 (Pro^{3}C$^\delta$H$_2$ minor conformer), 47.0 (Pro^{3}C$^\delta$H$_2$ major conformer), 59.2 (Pro^{3}C$^\alpha$H major conformer), 60.0

[¶] This signal consisted of two overlapping triplets: δ_H 1.18 (t, *J* 7.2, minor conformer) and 1.18 (t, *J* 7.1, major conformer).

[*] This signal consisted of a doublet of doublets, δ_H 3.82 (dd, *J* 6.2 and 17.3) due to the major conformer, overlaping a multiplet due to the minor conformer.

[†] This signal consisted of two overlapping quartets: δ_H 4.08 (t, *J* 7.1, major conformer) and 4.09 (t, *J* 7.2, minor conformer).

(Pro^{3}C$^\alpha$H minor conformer), 60.3 (OCH_2CH_3 major conformer), 60.4 (OCH_2CH_3 minor conformer), 61.6 (br, Val ^{1}C$^\alpha$H minor conformer), 61.7 (Val ^{1}C$^\alpha$H major conformer), 77.7 [$(CH_3)_3C$], 154.5 (Boc CO), 165.2 (amide CO minor conformer), 165.6 (amide CO major conformer), 166.4 (amide CO major conformer), 166.6 (amide CO minor conformer), 169.5 (Gly$^4CO_2Et$), 171.9 (Pro^{3}CO major conformer) and 172.2 (Pro^{3}CO minor conformer); m/z 457 [$(M+H)^+$, 15%], 340 [100, (M+H) - CH_2CMe_2, H_2O, CO, NH], 286 [70, (M+H) - BocNCHPr^i], 155 {50, $H_2NCOCH_2CO\overline{N[CH_2]_3CH^+}$}, 72 (31, H_2NCHPr^{i+}), 70 {50, $H\overline{N[CH_2]_3CH^+}$}, and 57 (37, Bu^{t+}).

*(l) C-Terminal deprotection of Boc-Valψ(NHCO)Gly-Pro-Gly-OEt, **133**, by saponification*

2 M Sodium hydroxide (0.4 mL) was added to a solution of Boc-Valψ(NHCO)Gly-Pro-Gly-OEt, **133** (156 mg, 0.34 mmol) in ethanol (2.5 mL). The mixture was stirred at r.t. overnight, then diluted with water (5 mL) and acidified to pH 2/3 by careful addition of 1 M potassium hydrogen sulfate. Brine (7 mL) was added and the solution extracted with dichloromethane (5 x 15 mL). The combined organic extracts were dried over sodium sulfate, evaporated and dried under high vacuum to yield Boc-Valψ(NHCO)Gly-Pro-Gly, **134**, as a colourless solid (114 mg, 78%), m.p. 149-153°C (change in appearance noted at 135-145°C) (Found: C, 53.3; H, 7.65; N, 12.7. $C_{19}H_{32}N_4O_7$ requires C, 53.25; H, 7.55; N, 13.1%); $[\alpha]_D^{17}$ - 45.7 (*c* 0.99 in ethanol); ν_{max} /cm^{-1} 3305br,m (NH), 3050-2500br,w (CO_2H), 1755m (acid carbonyl), 1720m (acid carbonyl), 1690s (Boc carbonyl), 1650vs (amide carbonyl), 1625s (amide carbonyl), 1555m (amide II), 1510s (amide II), 1290w, 1250m, 1175m, 1045w, 1000w, 850w and 720m; δ_H(400 MHz, $DMSO_{d6}$) 0.81-0.86 [6 H, m, $CH(CH_3)_2$], 1.37 (9 H, s, Boc), 1.75-2.20 (5 H, m, $CH(CH_3)_2$, Pro^{3}C$^\beta$H$_2$ and C$^\gamma$H$_2$), 3.02 (0.4 H, d, *J* 15.6, Gly^{2}C$^\alpha$H$_2$ minor conformer), 3.16 (0.4 H, d, *J* 15.6, Gly^{2}C$^\alpha$H$_2$ minor conformer), 3.24 (0.9 H‡, d, *J* 16.1, Gly^{2}C$^\alpha$H$_2$ major conformer), 3.29 (1.1 H, s§ Gly^{2}C$^\alpha$H$_2$ major conformer), 3.40-3.60 (2 H, m, Pro^{3}C$^\delta$H$_2$), 3.67 (0.7 H, dd, *J* 5.9 and 17.6, Gly^{4}C$^\alpha$H$_2$ major conformer), 3.72-3.79 (1.1 H, m, Gly^{4}C$^\alpha$H$_2$ both conformers), 4.31 (0.7 H, dd, *J* 2.5 and 6.4, Pro^{3}C$^\alpha$H major

‡ Some overlap with the water signal.

§ Pressumably a doublet partially obscured by the water signal.

conformer), 4.53 (0.3 H, dd, *J* 2.5 and 8.3, $Pro^3C^{\alpha}H$ minor conformer), 4.97 (1 H, q, *J* 8.3, $Val^1C^{\alpha}H$), 6.98-7.09 (1 H, m, BocNH), 8.10-8.17 (1.6 H, m, $Val^1\psi NH$ and Gly^4NH), 8.43 (0.3 H, t, *J* 5.9, NH minor conformer) and 12.53 (1 H, br s, CO_2H); δ_C(100.4 MHz; $DMSO_{d6}$) 18.2 [$CH(CH_3)_2$ minor conformer], 18.3 [$CH(CH_3)_2$], 18.4 [$CH(CH_3)_2$], 22.4 ($Pro^3C^{\gamma}H_2$ minor conformer), 23.9 ($Pro^3C^{\gamma}H_2$ major conformer), 28.3 [$(CH_3)_3C$], 29.7 ($Pro^3C^{\beta}H_2$ major conformer), 31.6 ($Pro^3C^{\beta}H_2$ minor conformer), 32.1 [$CH(CH_3)_2$ major conformer], 32.2 [$CH(CH_3)_2$ minor conformer], 40.6 ($Gly^4C^{\alpha}H_2$ major conformer), 40.8 ($Gly^4C^{\alpha}H_2$ minor conformer), 41.9 ($Gly^2C^{\alpha}H_2$ minor conformer), 42.4 ($Gly^2C^{\alpha}H_2$ major conformer), 46.6 ($Pro^3C^{\delta}H_2$ minor conformer), 47.2 ($Pro^3C^{\delta}H_2$ major conformer), 59.4 ($Pro^3C^{\alpha}H$ major conformer), 60.1 ($Pro^3C^{\alpha}H$ minor conformer), 61.9 (br, $Val^1C^{\alpha}H$), 77.9 [$(CH_3)_3C$], 154.7 (Boc CO), 165.4 (amide CO minor conformer), 165.7 (amide CO major conformer), 166.5 (amide CO major conformer), 166.8 (amide CO minor conformer), 171.2 (Gly^4CO_2H), 171.9 (Pro^3CO major conformer) and 172.2 (Pro^3CO minor conformer); m/z 429 [$(M+H)^+$, 429.2360, $C_{19}H_{33}N_4O_7$ requires 429.2349, 24%], 393 (22), 322 (18), 312 [56, (M+H) - CH_2CMe_2, H_2O, CO, NH], 281 (13), 258 [36, (M+H) - BocNCHPr^i], 221 (16), 207 (14), 147 (42), 99 (22) and 73 (100).

(m) Coupling of Boc-Valψ(NHCO)Gly-Pro-Gly, ***134****, and TFA.Valψ(NHCO)Gly-OEt,* ***119***[242]

A solution of TFA.Valψ(NHCO)Gly-OEt, **119** (81 mg, 0.25 mmol) in DMF (1.5 mL) was prepared. A solution of Boc-Valψ(NHCO)Gly-Pro-Gly, **134** (99 mg, 0.23 mmol) in DMF (1.0 mL) was cooled to -15°C in an inert atmosphere. *N*-Methylmorpholine (25 μL, 23 mg, 0.23 mmol) and *iso*-butylchloroformate (30 μL, 32 mg, 0.23 mmol) were successively added and stirring continued at -15°C, in an inert atmosphere, for 30 m.

Triethylamine (36 μL, 26 mg, 0.25 mmol) was added to the solution of TFA.Valψ(NHCO)Gly-OEt, **119**, and the resultant mixture immediately added to the solution of Boc-Valψ(NHCO)Gly-Pro-Gly, **134**, *N*-methylmorpholine and *iso*-butylchloroformate. Stirring was continued at -15°C for 30 m, and then at r.t. overnight, in an inert atmosphere. The solution was evaporated to produce a yellow oil, to which

water (7.5 mL) was added. The resultant off-white precipitate was collected, washed with water (4 mL) and dried under high vacuum over phosphorus pentoxide. Further crude product was recovered from the filtrate by extraction with dichloromethane (4 x 15 mL). The combined organic extracts were dried over sodium sulfate and evaporated. The two portions of crude product were combined and column chromatography (sample applied as a solution in the initial eluent), with chloroform / methanol / acetic acid (95:3:2 then 90:8:2) as the eluent, yielded:

ethyl malonamate, **118**, as a colourless oil (6.3 mg, 19%), identified by ^{1}H NMR;
and Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1** (26.0 mg, 18%). This material was further purified by dissolving in hot ethanol and collecting the colourless solid which precipitated on cooling, to yield Boc-Valψ(NHCO)Gly-Pro-Gly-Valψ(NHCO)Gly-OEt, **1** (7.7 mg, 5%); m.p. 203-207°C dec; $[\alpha]_D^{19}$ - 10.6 (*c* 0.51 in TFE); ν_{max} /cm^{-1} 3295br,m (NH), 1745m (ester carbonyl), 1735w (ester carbonyl), 1715w (ester carbonyl), 1695vs (Boc carbonyl), 1660vs (amide carbonyl), 1650vs (amide carbonyl), 1560s (amide II), 1540m (amide II), 1515br,m (amide II), 1505m (amide II), 1290w, 1245m, 1225m, 1170m, 1150m, 1075w, 1035w, 1015w, 965w, 865w and 720m; δ_H(400 MHz, DMSO$_{d6}$) 0.77-0.87 [12 H, m, CH(C*H*$_3$)$_2$], 1.17 (3 H, t, *J* 7.2, OCH$_2$C*H*$_3$), 1.37 (9 H, s, Boc), 1.69-2.20 (6 H, m, C*H*(CH$_3$)$_2$, Pro^{3}C$^\beta$H$_2$ and C$^\gamma$H$_2$), 3.04-3.33 (4 H, m, Gly^{2}C$^\alpha$H$_2$ and Gly^{6}C$^\alpha$H$_2$), 3.37-3.58 (3 H¶, m, Pro^{3}C$^\delta$H$_2$), 3.58-3.86 (2 H, m, Gly^{4}C$^\alpha$H$_2$), 4.06 (2 H, q, *J* 7.1, OC*H*$_2$CH$_3$), 4.26 (0.7 H, br dd, *J* 3.7 and 8.3, Pro^{3}C$^\alpha$H major conformer), 4.49 (0.3 H, br dd, *J* 3.0 and 9.0, Pro^{3}C$^\alpha$H minor conformer), 4.91-5.01 (1 H, m, Val^{1}C$^\alpha$H), 5.17-5.28 (1 H, m, Val^{5}C$^\alpha$H), 6.97-7.10 (1 H, m, BocNH), 7.66-7.73 (0.7 H, m, Val^{5}NH* major conformer), 8.09-8.19 (1.7 H, m, Val^{1}ψNH and Val^{5}NH*) and 8.24-8.33 (1.3 H, m, Gly^{4}NH and Val^{5}NH* minor conformer); δ_C(100.4 MHz; DMSO$_{d6}$) 14.1 (OCH$_2$*C*H$_3$), 17.9 [CH(*C*H$_3$)$_2$ minor conformer], 18.1 [CH(*C*H$_3$)$_2$ minor conformer], 18.3 [CH(*C*H$_3$)$_2$], 18.4 [CH(*C*H$_3$)$_2$], 24.3 (Pro^{3}C$^\gamma$H$_2$ major conformer), 28.3

¶ Due to overlap with the water signal.

* Unambiguous assignment of Val^{5}NH and Val^{5}ψNH was not possible, therefore in the above ^{1}H NMR data, "Val^{5}NH" means Val^{5}NH and / or Val^{5}ψNH.

[(CH_3)$_3$C], 29.4 (Pro^{3}C$^\beta$H$_2$ major conformer), 31.8 [CH(CH$_3$)$_2$], 32.0 [CH(CH$_3$)$_2$], 42.1 (Gly^{4}C$^\alpha$H$_2$), 42.5 (Gly^{2}C$^\alpha$H$_2$ and Gly^{6}C$^\alpha$H$_2$), 47.3 (Pro^{3}C$^\delta$H$_2$ major conformer), 59.9 (Pro^{3}C$^\alpha$H major conformer), 60.2 (ValC$^\alpha$H major conformer), 60.3 (Pro^{3}C$^\alpha$H minor conformer), 60.3 (ValC$^\alpha$H minor conformer), 60.5 (OCH_2CH$_3$), 61.9 (ValC$^\alpha$H minor conformer), 78.0 [(CH$_3$)$_3$C], 154.7 (Boc CO), 164.6 (CO), 165.8 (CO), 166.9 (CO), 167.9 (CO), 168.0 (CO), 172.0 (Pro^{3}CO major conformer) and 172.2 (Pro^{3}CO minor conformer); m/z 1247 [(2M+Na)$^+$, 12%], 635 [100, (M+Na)$^+$], 613 [11, (M+H)$^+$, 613.3523, $C_{28}H_{49}N_6O_9$ requires 613.3561], 496 [23, (M+H) - CH_2CMe_2, H_2O, CO, NH], 464 [19, (M+Na) - BocNCHPr^i], 442 [11, (M+H) - BocNCHPr^i], 393 (29), 365 [17, 496 - HNC(OH)CH$_2$CO$_2$Et], 333 (14), 322 (30), 311 (42, 496 - Pr^{i}CHNCOCH$_2$CO$_2$Et], 294 (27), 257 [25, 442 - Pr^{i}CHNCOCH$_2$CO$_2$Et], 240 (30, H$_2$NCOCH$_2$CO-Pro-NHCH$_2$CO$^+$), 186 [12, Pr^{i}CHNHCOCH$_2$CO$_2$Et$^+$], 183 (17), 116 (10) and 72 (75, H$_2$NCHPr^{i+}).

(n) N-*Terminal deprotection of Boc-Valψ(NHCO)Gly,* ***121****, with TFA.*

TFA (2.6 mL, 3.8 g, 34 mmol) was added to a solution of Boc-Valψ(NHCO)Gly, **121** (704 mg, 2.6 mmol) in dichloromethane (4.3 mL). The mixture was stirred at r.t. in a dry atmosphere for 40 m, after which time TLC (mixture E) indicated all the starting material, **121**, was consumed. The solution was evaporated to dryness, petrol (25 mL) added to the residue and the resultant mixture slowly stirred overnight. The petrol was decanted off and the process repeated with a further aliquot of petrol (25 mL). Ether (25 mL) was added to the residue and the resultant off-white suspension slowly stirred overnight. The off-white precipitate was collected by filtration in an inert atmosphere, dissolved in ethanol and evaporated to furnish a yellow solid. Addition of ether (2 x 20 mL) to the solid, followed by evaporation and drying under high vacuum yielded crude TFA.Valψ(NHCO)Gly, **142**, as a colourless solid (604 mg, 82%), m.p. 94-100°C dec; $[\alpha]_D^{23}$ + 24.2 (*c* 2.89 in ethanol); ν_{max} /cm^{-1} 3500-2500br,vs (CO_2H and NH_3^+), 3285s (NH), 1720s (acid carbonyl), 1665br,vs (carboxylate and amide carbonyl), 1545s (amide II), 1520s (NH bend), 1315m, 1195br,vs (C-F and / or C-O), 1145br,vs (C-F and / or C-O), 1065m, 985w, 950m, 915w, 865m, 840s, 800s, 720s (C-F), 660m (C-F) and 600w;

δ_H(270 MHz; $DMSO_{d6}$; TMS)† 0.94 [6 H, d, *J* 6.6, $CH(CH_3)_2$], 2.01 [1 H, octet, *J* 6.8, $CH(CH_3)_2$], 3.19 (1 H, d, *J* 15.4, $COCH_2CO$), 3.30 (1 H, d, *J* 15.4, $COCH_2CO$), 4.71 [1 H, t, *J* 7.9, $HN(CHPr^i)NH$], 8.07 (4 H, br s, ^+H_3N and CO_2H) and 9.01 (1 H, d, *J* 8.4, NH); δ_C(100.4 MHz; $DMSO_{d6}$)‡ 17.0 [$CH(CH_3)_2$], 18.1 [$CH(CH_3)_2$], 30.5 [$CH(CH_3)_2$], 42.6 ($COCH_2CO$), 61.6 [$HN(CHPr^i)NH$], 117.2 (q, *J* 300, $CF_3CO_2^-$), 158.5 (q, *J* 31, $CF_3CO_2^-$), 167.0 (amide CO) and 169.2 (acid CO); m/z 349 [$(2M-H)^+$, 15%], 175 (76, M^+, 175.1071, $C_7H_{15}N_2O_3$ requires 175.1083), 158 (100, M - NH_3, 158.0814, $C_7H_{12}NO_3$ requires 158.0817), 131 (24, M - CO_2), 110 (10) and 72 (84, $Pr^iCHNH_2^+$); m/z (-ve) 340 [$(173+matrix)^-$, 33%], 266 [59, $(CF_3CO_2+NBA)^-$], 173 [41, $H_2N(CHPr^i)NHCOCH_2CO_2^-$] and 113 (100, $CF_3CO_2^-$).

(o) N-*Terminal protection of TFA.Valψ(NHCO)Gly,* ***142****, with Fmoc*[333]

TFA.Valψ(NHCO)Gly, **142**, (516 mg, 1.8 mmol) and sodium hydrogen carbonate (300 mg, 3.6 mmol) were dissolved in a mixture of water (5.5 mL) and acetone (5.5 mL) with a concomitant evolution of gas. 9-Fluorenylmethyloxycarbonyl succinimide (604 mg, 1.8 mmol) was added and the cloudy mixture stirred overnight at r.t., after which time TLC (mixture E) indicated complete consumption of the 9-fluorenylmethyloxycarbonyl succinimide. The mixture was diluted with acetone and acidified to pH 2 with 2 M hydrochloric acid. The solution was concentrated *in vacuo*, the precipitate collected, washed with water (65 mL) and dried under high vacuum over phosphorus pentoxide. Column chromatography (sample applied as a slurry in the eluent), with chloroform / methanol / acetic acid (95:3:2) as eluent yielded:

9-fluorenylmethyl carbamate (Fmoc-NH_2), **145**, as a colourless solid (75 mg, 17%), m.p. 195-199°C (lit., 192-195°C[327], 200-201°C[385]); ν_{max} /cm^{-1} 3430m (NH), 3320w (NH), 3260w (NH), 3205w (NH), 1685vs (Fmoc CO), 1615s (amide II), 1425s, 1335s, 1105m, 1090m, 1055s, 800w, 760s and 740s; δ_H(270 MHz; $DMSO_{d6}$) 4.17-4.28 (3 H, m, CH and

† The crude material's ^1H NMR spectrum also contained the following weak signals, due to impurities: δ_H 3.09 (0.3 H, s), 7.10 (br s), 7.19 (br s), 7.57 (br m) and 7.71 (br s).

‡ The crude material's ^{13}C NMR spectrum also contained the following weak signals, due to impurities: δ_C 15.3, 19.2, 42.5, 65.0 and 169.6.

CH_2), 6.54 (1 H, br s, NH_2), 6.75 (1 H, br s, NH_2), 7.33 (2 H, t, *J* 7.1, Ar CH), 7.41 (2 H, t, *J* 7.3, Ar CH), 7.69 (2 H, d, *J* 7.3, Ar CH) and 7.89 (2 H, d, *J* 7.3, Ar CH) [lit.,[327] 4.25 (3 H, apparent s), 6.6 (2 H, br, NH_2), 7.34 (2 H, t, *J* 7.3), 7.43 (2 H, t, *J* 7.2), 7.70 (2 H, d, *J* 7.0) and 7.90 (2 H, d, *J* 6.8)]; m/z (C.I.) 257 [$(M+NH_4)^+$, 2%], 240 [2, $(M+H)^+$], 239 (1, $M^{\bullet+}$), 196 (14, 9-fluorenylmethanol radical cation), 180 (22), 179 (100, 9-fluorenylmethyl cation), 178 (42, dibenzofulvene radical cation), 166 (10), 165 (14), 71 (12), 69 (19) and 67 (16) [lit.,[327] m/z (E.I., source 200°C, solid probe 175°C, 20 eV) 239 [($M^{\bullet+}$, 2%], 196 (15, M - HNCO), 178 (100, M - CO_2, NH_3), 166 (37) and 165 (43)];

and crude Fmoc-Valψ(NHCO)Gly, **144**, contaminated with *N*-hydroxysuccinimide (353 mg); crystallisation from acetone / water yielded Fmoc-Valψ(NHCO)Gly, **144**, as a colourless solid (217 mg, 31%), m.p. 207-210°C; $[\alpha]_D^{19}$ + 10.7 (*c* 0.61 in DMSO); ν_{max} /cm^{-1} 3290s (NH), 1730m (acid carbonyl), 1715m (acid carbonyl), 1695br,vs (Fmoc CO), 1650s (amide carbonyl), 1635w (Ar CH), 1625w (Ar CH), 1565m (Ar CH), 1560m (amide II), 1540w (amide II), 1520s (amide II), 1505m (Ar CH), 1280w, 1255s, 1145w, 1125w, 1085w, 1080w, 1030s, 965w, 795w, 760w and 740m; δ_H(400 MHz; $DMSO_{d6}$) 0.86 [6 H, d, *J* 6.6, $CH(CH_3)_2$], 1.82-1.93 [1 H, m, $CH(CH_3)_2$], 3.10 (1 H, d, *J* 15.4, $COCH_2CO$), 3.20 (1 H, d, *J* 15.4, $COCH_2CO$), 4.19-4.33 (3 H, m, Fmoc CH and CH_2), 5.00 [1 H, br q, *J* 8.1, $HN(CHPr^i)NH$], 7.30-7.35 (2 H, m, Fmoc Ar CH), 7.41 (2 H, t, *J* 7.5, Fmoc Ar CH), 7.64 (1 H, br d, *J* 8.4, FmocNH), 7.71 (2 H, br d, *J* 6.6, Fmoc Ar CH), 7.89 (2 H, d, *J* 7.3, Fmoc Ar CH), 8.20 (1 H, br d, *J* 7.7, NH) and 12.50 (br s, CO_2H); δ_C(100.4 MHz; $DMSO_{d6}$) 18.4 [$CH(CH_3)_2$], 18.5 [$CH(CH_3)_2$], 32.0 [$CH(CH_3)_2$], 42.6 ($COCH_2CO$), 46.8 (Fmoc CH), 62.4 [$HN(CHPr^i)NH$], 65.4 (Fmoc CH_2), 120.2 (Fmoc Ar CH), 125.4 (Fmoc Ar CH), 127.1 (Fmoc Ar CH), 127.7 (Fmoc Ar CH), 140.8 (Fmoc Ar C), 143.9 (br, Fmoc Ar C), 155.3 (Fmoc CO), 165.2 (amide CO) and 169.6 (acid CO); m/z 410 (13%), 397 [48, $(M+H)^+$, 397.1759, $C_{22}H_{25}N_2O_5$ requires 397.1763], 368 (53), 294 (22, $FmocNHCHPr^{i+}$), 191 (62), 179 (100, 9-fluorenylmethyl cation), 178 (96, dibenzofulvene radical cation), 158 (79, $Pr^iCHNHCOCH_2CO_2H^+$), 147 (37), 133 (29), 117 (23), 94 (29) and 73 (65).

3.2.4 *Phe series.*

(a) Preparation of Boc-Pheψ(NHCO)Gly-OEt, ***116(b)***

Boc-phenylalanine **113(b)** (2.0 g, 7.5 mmol) was dissolved in THF (15 mL) and cooled to 0°C in an inert atmosphere. Tri-*n*-butyl amine (1.4 g, 1.8 mL, 7.5 mmol) followed by ethyl chloroformate (0.82 g, 0.72 mL, 7.6 mmol) were added slowly, with stirring and the temperature maintained at 0°C. Stirring was continued at 0°C for 30 m. An ice cold solution of sodium azide (0.98 g, 15 mmol) in the minimum volume of water (2.4 mL) was added and stirring continued for 1 h at 0°C. Ice cold portions of ethyl acetate (50 mL) and sat. sodium hydrogen carbonate (50 mL) were added and the mixture shaken and separated. The organic portion was washed with ice cold brine (50 mL), dried over magnesium sulfate and evaporated at <25°C.§ The resultant colourless solid was dissolved in toluene (15 mL): IR analysis at this stage indicated a mixture of acyl azide, **114(b)**, [ν_{max}(tol) 2140 cm^{-1}] and isocyanate, **115(b)**, [ν_{max}(tol) 2250 cm^{-1}]. The solution was heated to 70°C in an inert atmosphere, with stirring, for *ca.* 40 m (during which vigorous effervescence occurred), until IR analysis indicated all the acyl azide, **114(b)**, [ν_{max}(tol) 2140 cm^{-1}] had converted to the isocyanate, **115(b)**, [ν_{max}(tol) 2250 cm^{-1}]. The solution was maintained at 70°C and monoethyl malonate, **98** (1.2 g, 9.1 mmol) added. Stirring was continued at 70°C for *ca.* 10 m (during which further effervescence occurred), after which time IR analysis indicated all the isocyanate, **115(b)**, was consumed. The solution was cooled to r.t. and petrol (50 mL) added. The resultant colourless precipitate was filtered off and dried under high vacuum.¶ Column

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§ When the flask was removed from the rotary evaporator, air was readmitted *via* a calcium chloride / silica gel drying tube.

¶ The first time this reaction was attempted, the addition of petrol produced very little precipitate, therefore the reaction mixture was evaporated to dryness. The resultant yellow oil was purified by column chromatography (sample preabsorbed from ethyl acetate solution) with ethyl acetate / petrol (10:90) as eluent and yielded *N*-(Boc)-1-amino-*trans*-styrene [*N*-(Boc)-2-phenyl-*trans*-ethenamine], **24(b)**, as a colourless solid, in 1% yield, m.p. 133-139°C; ν_{max} /cm^{-1} 3300s (NH), 1680vs (Boc carbonyl), 1655vs (alkene), 1510s (amide II), 1480s (Ph), 1330w, 1315s, 1300s, 1280s, 1240s, 1160vs, 1050m, 1020w, 950m, 850m, 740m and 680m; δ_H (270 MHz; $DMSO_{d6}$) 1.45 (9 H, s, Boc), 5.98 (1 H, d, *J* 14.7,CHC*H*Ph), 6.99-7.31 (6 H, m, Ph and BocNHC*H*) and 9.54 (1 H, br d, *J* 13.3, BocNH); δ_C (100.4 MHz; $CDCl_3$) 28.2

chromatography (sample preabsorbed from ethyl acetate solution*), with ethyl acetate / petrol (50:50) as eluent yielded Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, as a colourless crystalline solid (0.52 g, 20%), m.p. 115-117°C (from toluene / petrol) (Found: C, 61.5; H, 7.5; N, 8.15. $C_{18}H_{26}N_2O_5$ requires C, 61.7; H, 7.5; N, 8.0%); $[\alpha]_D^{23}$ + 7.6 (*c* 1.18 in deuterochloroform); ν_{max} /cm^{-1} 3335s (NH), 1745s (ester carbonyl), 1695vs (Boc carbonyl), 1650m (amide carbonyl), 1540m (amide II), 1510s (amide II), 1400w, 1340w, 1290w, 1270m, 1250m, 1225w, 1200w, 1170m, 1140m, 1065w, 1050m, 1015m, 950w, 940w, 880w and 855w; δ_H(270 MHz; $DMSO_{d6}$) 1.17 (3 H, t, *J* 7.2, OCH_2CH_3), 1.32 (9 H, s, Boc), 2.88 [2 H, br d, *J* 7.5, HN(CHCH_2Ph)NH], 3.20 (2 H, s, $COCH_2CO$), 4.05 (2 H, q, *J* 7.1, OCH_2CH_3), 5.25 [1 H, quintet, *J* 7.5, HN(CHCH$_2$Ph)NH], 7.19-7.30 (6 H, m, Ph and BocNH) and 8.39 (1 H, d, *J* 7.5, NH); δ_C(100.4 MHz; $CDCl_3$; TMS) 14.0 (OCH_2CH_3), 28.3 [(CH_3)$_3$C], 39.8 [HN(CHCH$_2$Ph)NH], 41.6 ($COCH_2CO$), 59.7 [HN(CHCH$_2$Ph)NH], 61.6 (OCH_2CH_3), 80.1 [(CH_3)$_3$C], 126.9 (Ph C-4), 128.6 (Ph C-3

[(CH_3)$_3$C], 80.9 [(CH_3)$_3$C], 109.6 (CHCHPh), 124.2 (Ph C-4), 125.1 (Ph C-3 or 2), 126.0 (BocNHCH), 128.6 (Ph C-2 or 3), 136.5 (Ph C-1) and 152.7 (Boc CO); m/z (E.I.) 219 (M^+, 219.12742, $C_{13}H_{17}NO_2$ requires 219.12593, 9%), 163 (59, M - CH_2CMe_2), 145 (10), 119 (58, 163 - CO_2), 118 (30), 91 (16), 59 (23), 57 (100, Bu^{t+}), 41 (37) and 29 (17, CHO^+). *N*-(Boc)-1-amino-*trans*-styrene, **24(b)**, was identified by TLC as a by-product in subsequent, more successful, syntheses of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**.

* The material which would not dissolve in ethyl acetate was filtered off and yielded crude *N*,*N*'-bis[(*S*)-1-(*N*-Boc-amino)-2-phenylethyl]-urea, **117(b)**, as a colourless solid. This material was very difficult to purify, but a relatively pure sample was obtained by exhaustive washing with methanol; ν_{max} /cm^{-1} 3340m (NH), 3215s (NH), 3065br,s (NH), 1780m, 1755m, 1710vs (Boc carbonyl), 1695vs (Boc carbonyl), 1650m (urea carbonyl), 1565w (amide II), 1530w (amide II), 1500w (Ph), 1420s, 1400s, 1305w, 1265w, 1240w, 1225w, 1170m, 1050m, 1025w, 850w, 790w and 750w; δ_H(270 MHz; $DMSO_{d6}$) 1.32 (9 H, s, Boc), 2.84 [2 H, br d, *J* 7.5, HN(CHCH_2Ph)NH], 5.08 [1 H, quintet, *J* 7.5, HN(CHCH$_2$Ph)NH], 6.41 (1 H, br d, *J* 7.5, BocNH) 7.15-7.30 (6 H, m, Ph and NH) and 10.91 (3 H, br s, impurity acid H); δ_C(67.8 MHz; $DMSO_{d6}$) 28.2 [(CH_3)$_3$C], 41.0 [HN(CHCH$_2$Ph)NH], 59.5 [HN(CHCH$_2$Ph)NH], 77.8 [(CH_3)$_3$C], 126.2 (Ph C-4), 128.1 (Ph C-3 or 2), 129.0 (impurity), 129.2 (Ph C-2 or 3), 138.0 (Ph C-1), 150.0 (impurity), 154.5 (CO) and 155.6 (CO); m/z 499 [$(M+H)^+$, 18%), 382 [21, (M+H) - CH_2CMe_2, H_2O, CO, NH], 326 (10, 382 - CH_2CMe_2), 280 [17, (M+H) - BocNCHCH$_2$Ph], 265 (38, 326 - H_2O, CO, NH), 224 (11, 280 - CH_2CMe_2), 220 (18, BocNHCHCH$_2$Ph$^+$), 186 (100), 164 (28, 220 - CH_2CMe_2), 163 (33, $H_2NCONHCHCH_2Ph^+$) and 120 (90, $PhCH_2CHNH_2^+$). This urea, **117(b)**, is thought to be responsible for the turbidity which sometimes developed in the toluene solution of the acyl azide, **114(b)** / isocyanate, **115(b)**, prior to the addition of monoethyl malonate, **98**.

or 2), 129.4 (Ph C-2 or 3), 136.6 (Ph C-1), 154.8 (Boc CO), 164.8 (CO) and 168.8 (CO); m/z 351 [$(M+H)^+$, 22%], 295 [19, (M+H) - CH_2CMe_2], 234 (79, 295 - H_2O, CO, NH), 203 (13), 164 (20), 132 [23, (M+H) - $BocNCHCH_2Ph$] and 120 (100, $PhCH_2CHNH_2^+$).

(b) N-*Terminal deprotection of Boc-Pheψ(NHCO)Gly-OEt,* ***116(b)****, with TFA (in the absence of triethylsilane)*

TFA (0.47 mL, 0.70 g, 6.1 mmol) was added to a solution of Boc-Pheψ(NHCO)Gly-OEt, **116(b)** (163 mg, 0.47 mmol) in dichloromethane (1.1 mL). The mixture was stirred at r.t. in a dry atmosphere for 90 m, after which time TLC (mixture B) indicated all the starting material, **116(b)**, was consumed. The mixture was evaporated to dryness and the resultant residue was triturated with ether. The resultant suspension was filtered, the precipitate washed with ether and dried under high vacuum. The filtrate was evaporated to dryness and further product obtained from the resultant residue by retrituration with ether, prolonged cooling (2 days) in a refridgerator, and filtration as before. Thus TFA.Pheψ(NHCO)Gly-OEt, **124**, (73 mg, 43%) was obtained as a hygroscopic, colourless solid, m.p. 91-94°C (Found: C, 49.4; H, 5.3; N, 8.0. $C_{15}H_{19}N_2O_5F_3$ requires C, 49.45; H, 5.25; N,7.7%); ν_{max} /cm^{-1} 3300m (NH), 3110br,s (NH), 2760s (NH_3^+), 2155br,w (NH_3^+), 1735vs (ester carbonyl), 1660br,vs (carboxylate and amide carbonyl), 1525s (amide II), 1500w (Ph), 1340s, 1210br,vs (C-F and / or C-O), 1185br,vs (C-F and / C-O), 1155vs (C-F and / C-O), 1080w, 1015m, 840w, 800m, 735m, 720s and 695m; δ_H(270 MHz; $DMSO_{d6}$) 1.13 (3 H, t, *J* 7.2, OCH_2CH_3), 2.99 [1 H, dd, J_{gem} 13.5, $^3J_{HH}$ 10.5, $HN(CHCH_2Ph)NH$], 3.06 [1 H, dd, J_{gem} 13.5, $^3J_{HH}$ 4.5, $HN(CHCH_2Ph)NH$], 3.25 (2 H, s, $COCH_2CO$), 4.02 (2 H, q, *J* 7.2, OCH_2CH_3), 5.09 [1 H, m, $HN(CHCH_2Ph)NH$], 7.13-7.38 (5 H, m, Ph), 8.41 (3 H, br s, NH_3^+), and 9.12 (1 H, d, *J* 8.3, NH); δ_C(67.8 MHz; $DMSO_{d6}$) 14.0 (OCH_2CH_3), 37.5 [$HN(CHCH_2Ph)NH$], 42.2 ($COCH_2CO$), 58.1 [$HN(CHCH_2Ph)NH$], 60.6 (OCH_2CH_3), 127.1 (Ph C-4), 128.5 (Ph C-3 or 2), 129.2 (Ph C-2 or 3), 135.2 (Ph C-1), 166.3 (CO) and 166.9 (CO); m/z 501 [$(2M-H)^+$, 14%], 251 (26, M^+), 234 (96, M - NH_3), 159 (10), 120 (100, $PhCH_2CHNH_2^+$) and 91 (10, $PhCH_2^+$); m/z (-ve) 266 [$(CF_3CO_2+NBA)^-$, 19%], 227 [49, $(2CF_3CO_2+H)^-$] and 113 (100, $CF_3CO_2^-$).

*(c) C-Terminal deprotection of Boc-Pheψ(NHCO)Gly-OEt, **116(b)**, by saponification*

2 M Sodium hydroxide (0.13 mL) was added to a solution of Boc-Pheψ(NHCO)Gly-OEt, **116(b)** (50 mg, 0.14 mmol) in ethanol (1.5 mL). The mixture was stirred at r.t. overnight, then diluted with water (10 mL) and concentrated *in vacuo*. Water (10 mL) was added and the solution washed with ethyl acetate (15 mL). The aq. portion was acidified to pH 2/3 by careful addition of 1 M potassium hydrogen sulfate. Brine (2 mL) was added and the solution extracted with ethyl acetate (4 x 10 mL). The organic extract was dried over sodium sulfate, evaporated and dried under high vacuum to yield Boc-Pheψ(NHCO)Gly, **125**, as a colourless solid (40 mg, 88%), m.p. 150-151°C (Found: C, 59.6; H, 6.95; N, 8.55. $C_{16}H_{22}N_2O_5$ requires C, 59.6; H, 6.9; N, 8.7%); $[\alpha]_D^{19}$ + 1.4 (*c* 0.43 in ethanol); ν_{max} /cm^{-1} 3335vs (NH), 3200-2500br,w (CO_2H), 1725s (acid carbonyl), 1690vs (Boc carbonyl), 1655m (amide carbonyl), 1555m (amide II), 1510m (amide II), 1380w, 1300m, 1270w, 1250m, 1230w, 1205m, 1175w, 1155m, 1065m, 1025m, 1000m, 940w, 915m, 865w and 835m; δ_H(270 MHz, $DMSO_{d6}$) 1.31 (9 H, s, Boc), 2.85 [2 H, br d, *J* 7.5, HN(CHC*H*$_2$Ph)NH], 3.12 (2 H, s, $COCH_2CO$), 5.26 [1 H, quintet, *J* 7.5, HN(C*H*CH$_2$Ph)NH], 7.18-7.27 (6 H, m, Ph and BocNH), 8.38 (1 H, d, *J* 7.5, NH); δ_C(67.8 MHz; $DMSO_{d6}$) 28.3 [(*C*H$_3$)$_3$C], 40.1 [HN(CH*C*H$_2$Ph)NH], 42.5 (CO*C*H$_2$CO), 58.9 [HN(*C*HCH$_2$Ph)NH], 78.1 [(CH$_3$)$_3$*C*], 126.3 (Ph C-4), 128.2 (Ph C-3 or 2), 129.3 (Ph C-2 or 3), 137.7 (Ph C-1), 154.4 (Boc CO), 165.1 (amide CO) and 169.6 (acid CO); m/z 667 [(2M+Na)$^+$, 5%], 645 [6, (2M+H)$^+$], 393 (13), 345 [20, (M+Na)$^+$], 323 [90, (M+H)$^+$], 267 [55, (M+H) - CH_2CMe_2], 231 (20), 206 (100, 267 - H_2O, CO, NH), 175 (25), 164 (22), 147 (12), 131 (17) and 73 (33).

*(d) Coupling of Boc-Pheψ(NHCO)Gly, **125**, and TFA.Pheψ(NHCO)Gly-OEt, **124***

A solution of TFA.Pheψ(NHCO)Gly-OEt, **124** (40 mg, 0.11 mmol), Boc-Pheψ(NHCO)Gly, **125** (33 mg, 0.10 mmol) and *N*-hydroxysuccinimide (12 mg, 0.10 mmol) in THF (1.0 mL) was cooled to -10°C in a dry atmosphere. EDC.HCl (21 mg, 0.11 mmol) and triethylamine (31 μL, 23 mg, 0.22 mmol) were successively added at -10°C, with stirring. The mixture was stirred at -10°C for 30 m, and then at r.t. for a further 7 days. The solution was evaporated to dryness and the resultant yellow residue dissolved

in dichloromethane (25 mL). The dichloromethane solution was washed successively with 5% citric acid (25 mL) and water (25 mL plus a few drops of brine). The dichloromethane layer was separated, dried over sodium sulfate, evaporated and dried under high vacuum. Column chromatography of the resultant orange residue (preabsorbed onto silica from a solution in dichloromethane), with chloroform / methanol / acetic acid (95:3:2) as eluent, followed by washing of the collected product with petrol and drying under high vacuum, yielded Boc-Pheψ(NHCO)Gly-Pheψ(NHCO)Gly-OEt, **126**, as a colourless solid (12 mg, 21%); m.p. 99-120°C dec; $[\alpha]_D^{19}$ + 5.5 (*c* 0.72 in DMSO); ν_{max} / cm^{-1} 3285s (NH), 1735w (ester carbonyl), 1725m (ester carbonyl), 1690vs (Boc carbonyl), 1680vs (amide carbonyl), 1565m (amide II), 1550m (amide II), 1530m (amide II), 1510s, 1500s (Ph), 1250w, 1170m, 1090w, 1045w and 1025w; δ_H(270 MHz, $DMSO_{d6}$) 1.15 (3 H, t, *J* 7.2, OCH_2CH_3), 1.31 (9 H, s, Boc), 2.84 (2 H, br d, *J* 7.5, Phe$^1C^\beta H_2$), 2.89 (2 H, d, *J* 6.6, Phe$^3C^\beta H_2$), 3.01 (2 H, s, Gly$C^\alpha H_2$), 3.18 (2 H, s, Gly$C^\alpha H_2$), 4.04 (2 H, q, *J* 7.2, OCH_2CH_3), 5.30 (1 H, quintet, *J* 7.5, Phe$^1C^\alpha H$), 5.49 (1 H, quintet, *J* 7.5, Phe$^3C^\alpha H$), 7.12-7.40 (11 H, m, Ph and BocNH), 8.40 (1 H, m, Phe$^1\psi$NH) and 8.63 (2 H, m, Phe^{3}NH and Phe$^3\psi$NH); δ_C(100.4 MHz; $DMSO_{d6}$) 14.1 (OCH_2CH_3), 28.2 [$(CH_3)_3C$], 40.9 [HN(CHCH_2Ph)NH], 41.0 [HN(CHCH_2Ph)NH], 42.4 ($COCH_2CO$), 42.6 ($COCH_2CO$), 57.4 [HN($CHCH_2$Ph)NH], 58.9 [HN($CHCH_2$Ph)NH], 60.5 (OCH_2CH_3), 77.9 [$(CH_3)_3C$], 126.3 (Ph C-4), 126.4 (Ph C-4), 128.2 (Ph C-3 or 2), 128.3 (Ph C-3 or 2), 129.3 (Ph C-2 or 3), 137.3 (Ph C-1), 137.6 (Ph C-1), 154.6 (Boc CO), 164.6 (CO), 165.9 (CO), 166.2 (CO) and 167.7 (CO); m/z 577 [$(M+Na)^+$, 100%], 555 [16, $(M+H)^+$, 555.2801, $C_{29}H_{39}N_4O_7$ requires 555.2819], 438 [23, (M+H) - CH_2CMe_2, H_2O, CO, NH], 393 (21), 358 [19, (M+Na) - BocNCHCH_2Ph], 336 [16, (M+H) - BocNCHCH_2Ph], 322 [23, BocNH(CHCH_2Ph)NHCOCH_2C(OH)NH_2^+], 234 (44, PhCH_2CHNHCO$CH_2CO_2Et^+$), 205 (66, $H_2NCOCH_2CONHCHCH_2Ph^+$), 176 (26), 164 (24), 132 [17, $H_2NC(OH)CH_2CO_2Et^+$] and 103 (17).

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Appendix

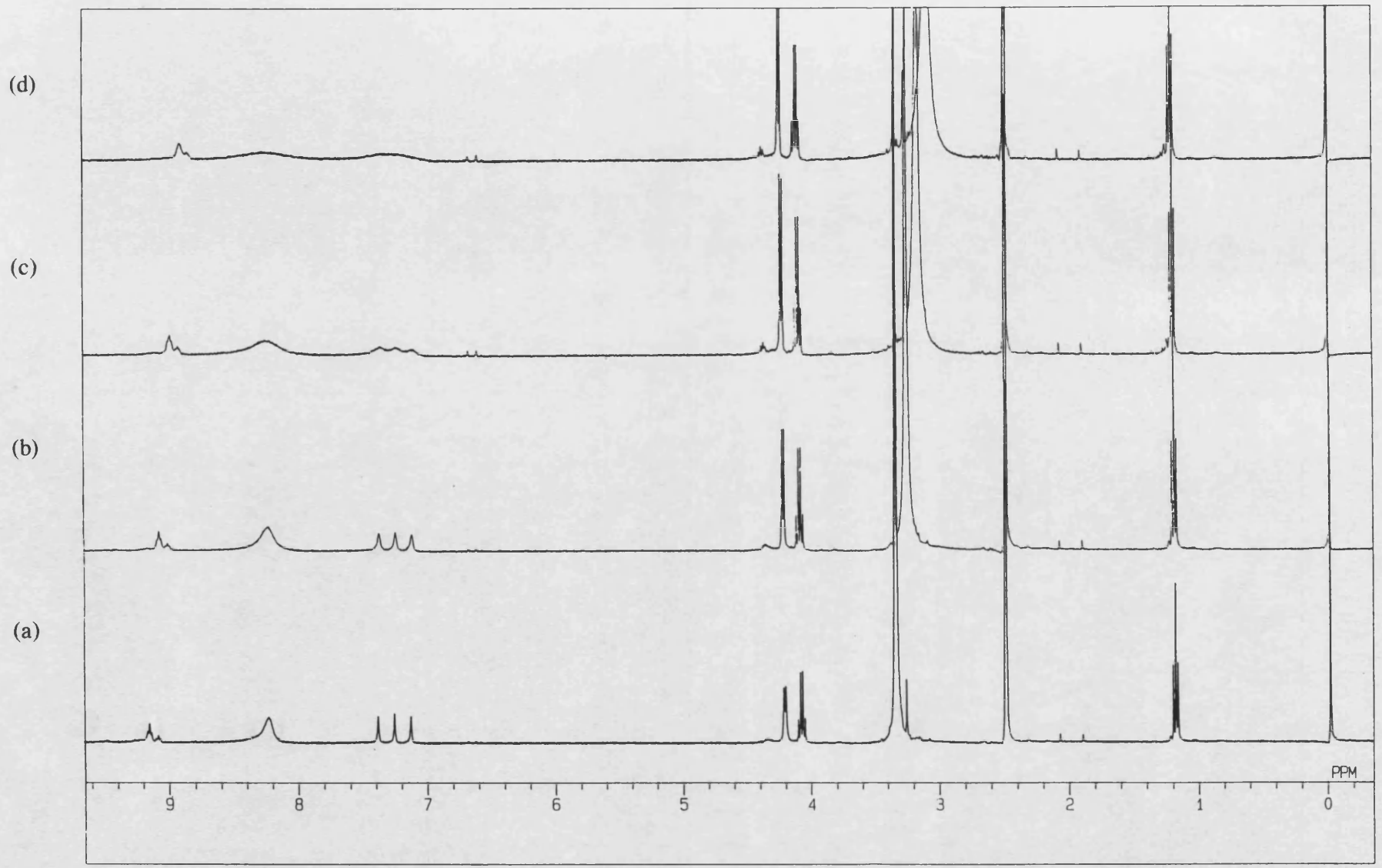


Figure A1: VT ^{1}H NMR study on HCl.Glyψ(NHCO)Gly-OEt, **101**. (a) 22°C, (b) 40°C, (c) 60°C, (d) 80°C, cont. over.

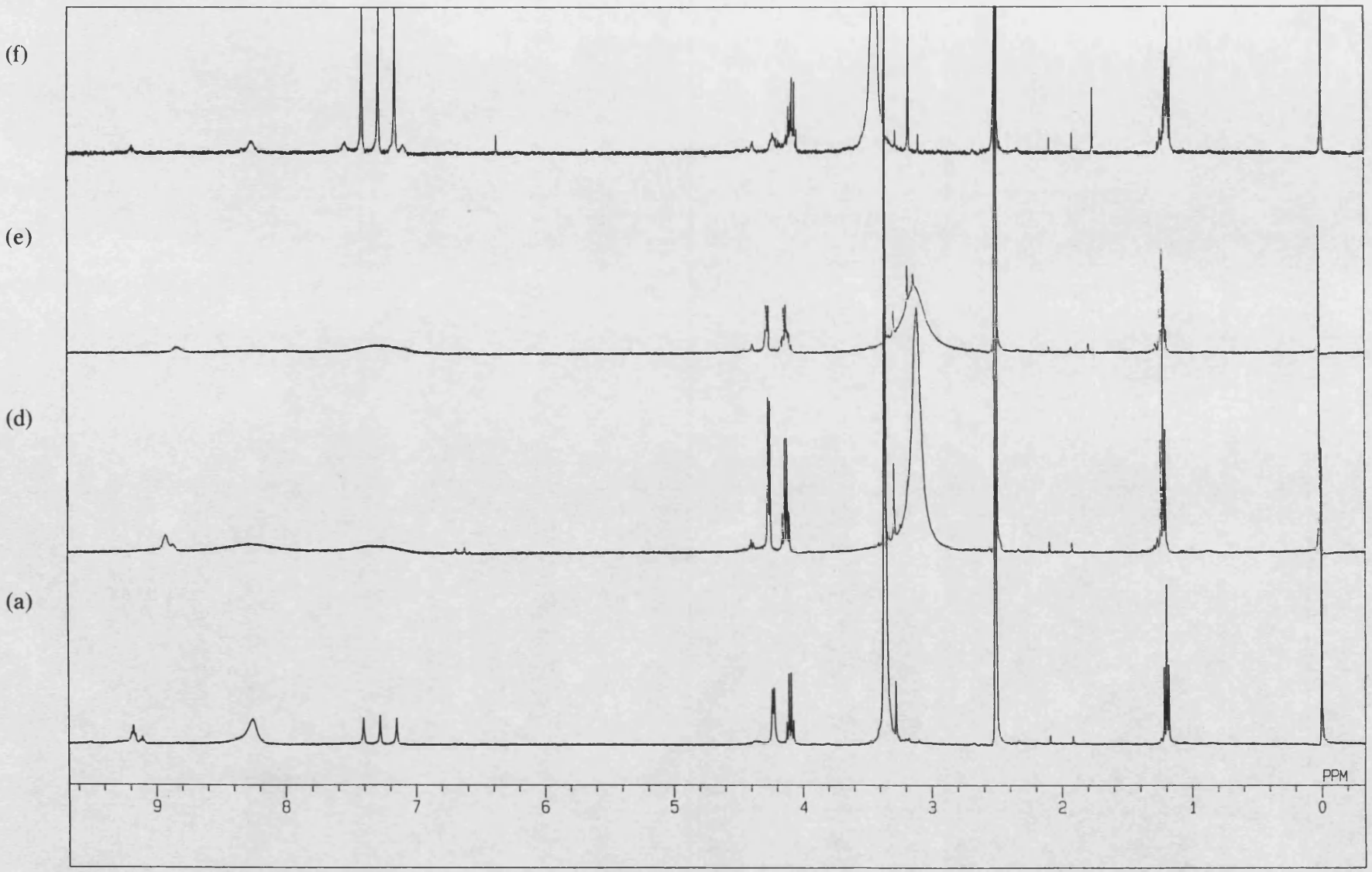


Figure A1 cont.: (a) 22°C, (d) 80°C, (e) 100°C, and (f) on return to r.t. (22°C). All in $DMSO_{d6}$.

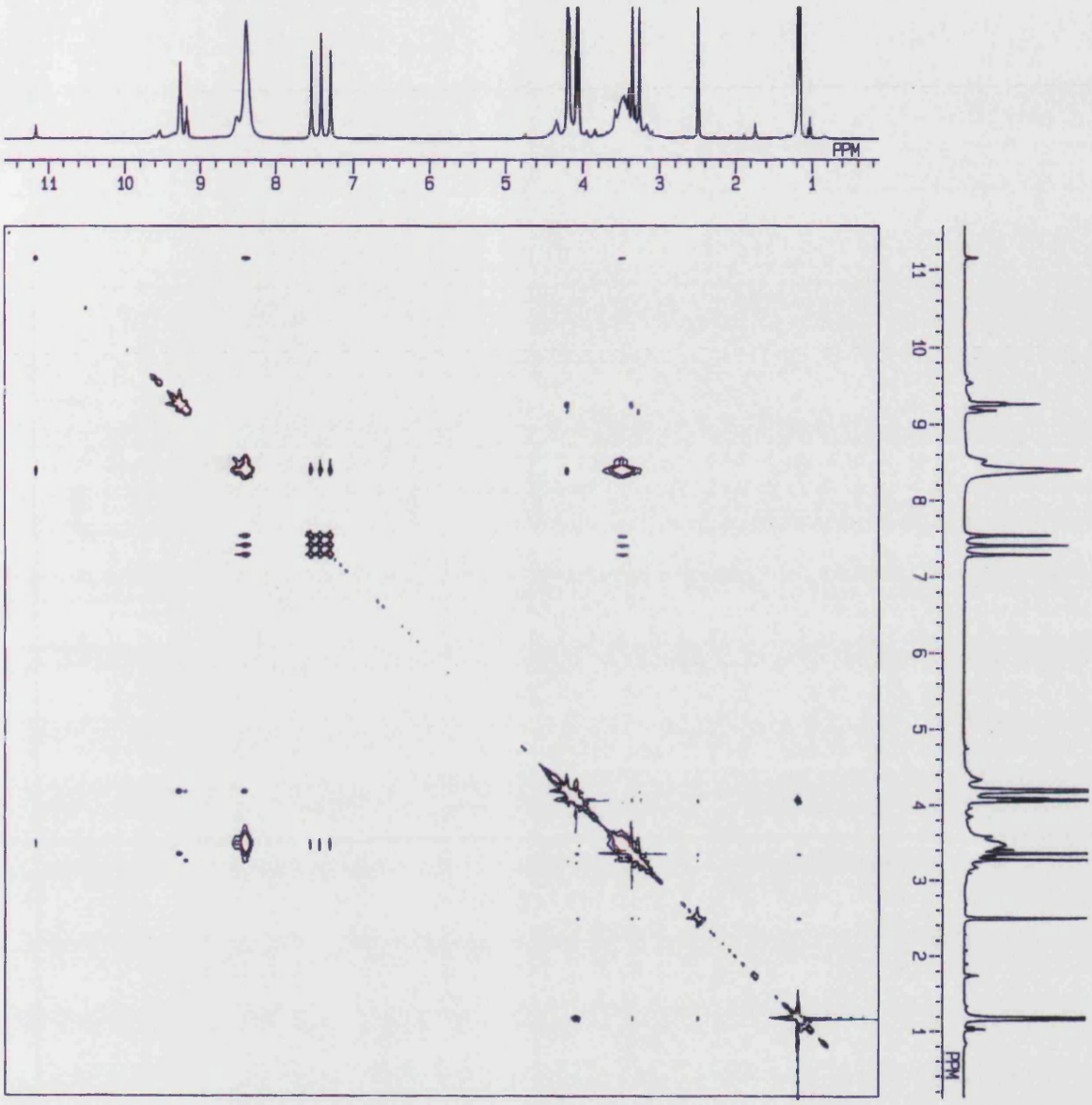


Figure A2: NOESY spectrum of HCl.Glyψ(NHCO)Gly-OEt, **101**.

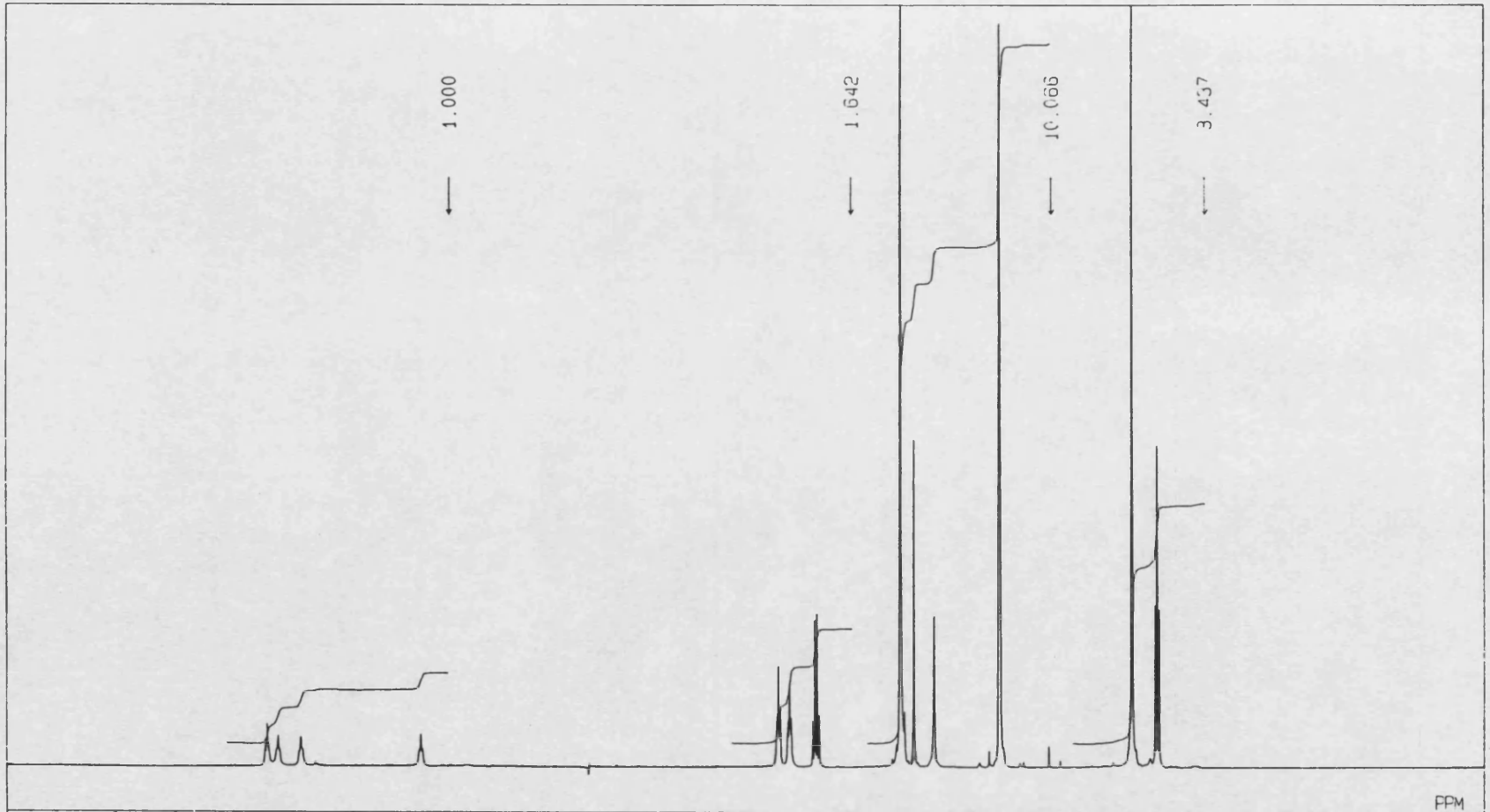


Figure A3(a): ^{1}H NMR spectrum of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** in $DMSO_{d6}$.

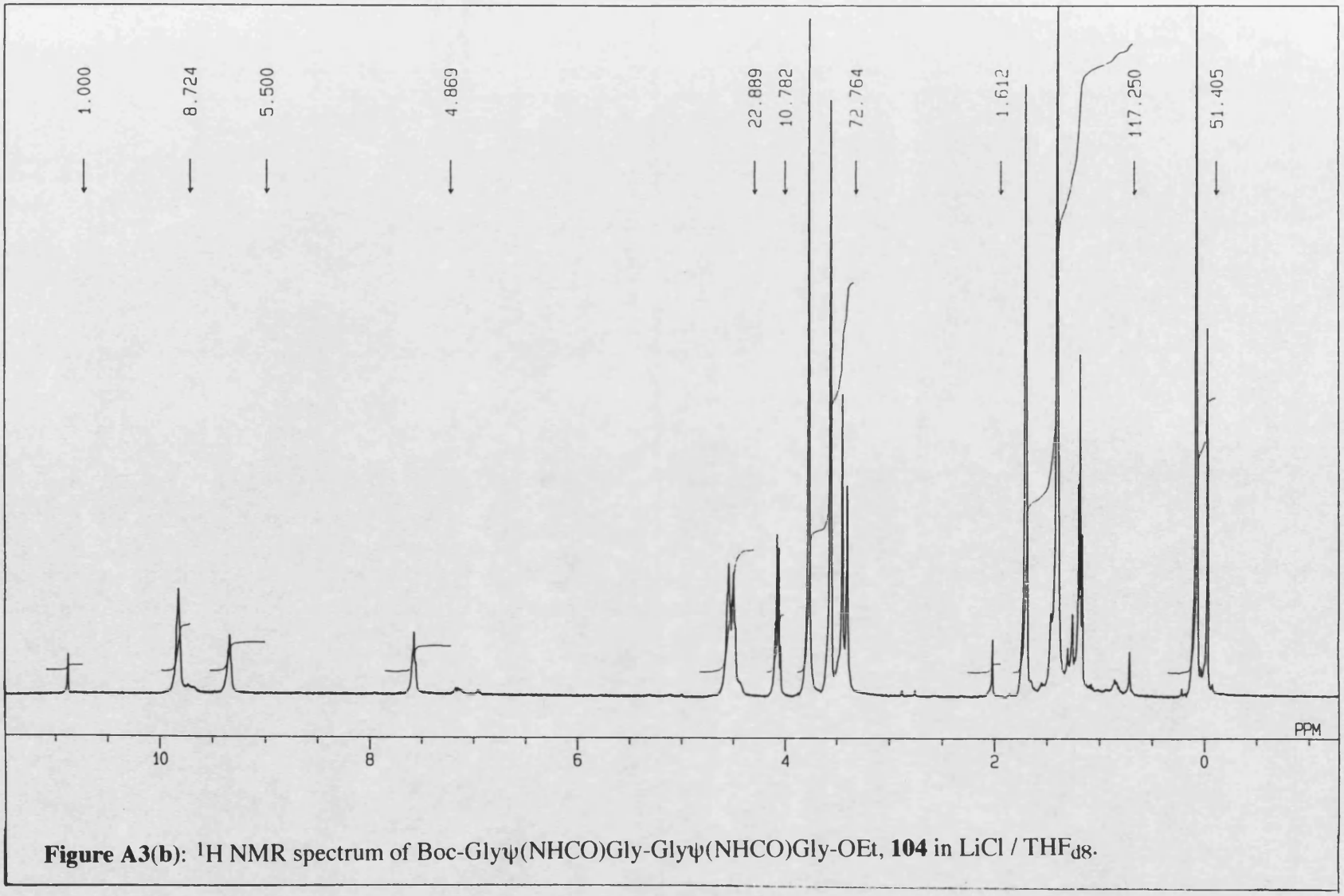
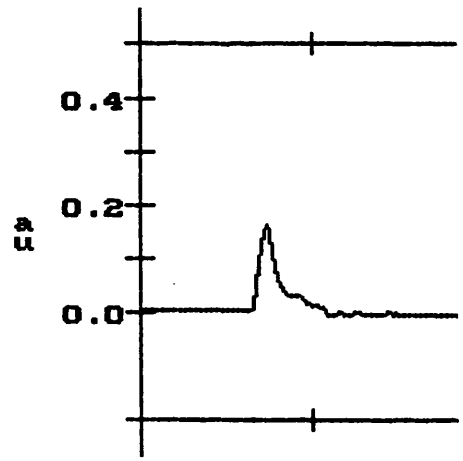
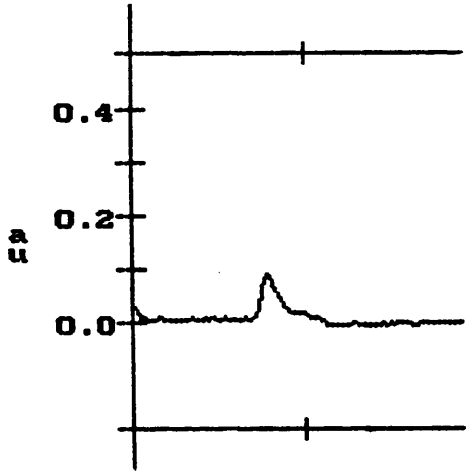


Figure A3(b): ^{1}H NMR spectrum of Boc-Glyψ(NHCO)Gly-Glyψ(NHCO)Gly-OEt, **104** in LiCl / THF_{d8}.



(a)



(b)

Figure A4: Fmoc deprotection profiles, corresponding to (a) step (i), and (b) step (xiii) of scheme 73.

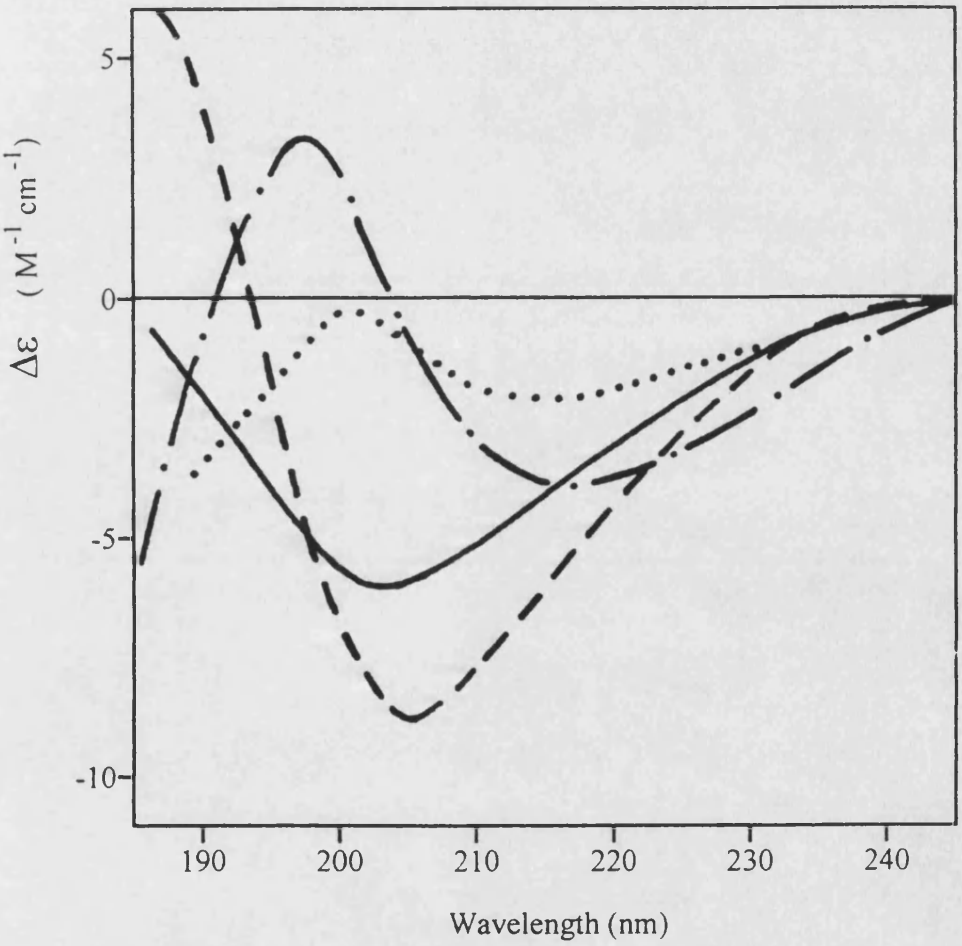


Figure A5: CD spectra of Boc-Glyψ(NHCO)Gly-Pro-Gly-Glyψ(NHCO)Gly-OEt, **132** as a function of solvent environment. Water: solid line. Supramicellar SDS 25 mM: dashed line. TFE: dashed-dot line. TFE / sodium hydroxide (57 mM): dotted line.